GUIDANCE OR PATTERNING? RESOLVING THE ROLE OF HEDGEHOG SIGNALING IN INTRARETINAL AXON PATHFINDING IN ZEBRAFISH

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

During formation of a functioning neuronal network, nerve processes have to travel long distances to reach their target tissue. Along their paths, axons are directed by guidance cues. For normal pathfinding, correct host tissue patterning is necessary to ensure the right set of guidance cues being expressed at the right place and time.

One of the wonders of nervous system development is how a small set of morphogens, regulating tissue patterning, and guidance molecules, acting directly on growth cones, lay out the tracks for the vast number of axons along their most diverse pathways in a complex nervous system like ours. Even more surprising, it has become evident that the same molecules act both in tissue patterning and as guidance cues, depending on the spatial and temporal context. One such molecule found in both roles is Sonic hedgehog (Shh).

The research presented in this dissertation used the zebrafish retinotectal system as a model to study the role of Shh in axon pathfinding. Zebrafish mutant for *shh* exhibit intraretinal axon pathfinding errors, where axons fail to exit the eye. Whether these errors are due to mispatterning of ocular tissue or the lack of direct guidance cues was not clear. We used temporal and spatial inhibition of the Hedgehog (Hh) signaling pathway to determine if Shh acts as a morphogen in tissue patterning or as guidance cue to regulate intraretinal axon pathfinding. Our results strongly suggest that Hh signaling regulates intraretinal pathfinding indirectly through tissue patterning. This is further supported by

the finding that several optic stalk markers are downregulated in *shh* mutants. We further investigated the role of one of these markers, *cxcl12a*, a chemokine known to be involved in axon guidance. Our analysis uncovered an interaction between the Hh and chemokine pathways for intraretinal axon pathfinding.

In summary, I determined that Hh signaling plays an indirect role in intraretinal axon pathfinding through patterning of the optic stalk and regulating the expression of the axon guidance molecule, *cxcl12a*, at the optic disc. These findings reveal a novel interaction between the Hh and chemokine pathways for the guidance of retinal axons out of the eye.

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CHAPTER 1

INTRODUCTION

Overview

In order to form a functioning nervous system, the developing neuronal tissue has to first be patterned correctly and then obtain the correct tissue identity. Second, differentiated neurons need to send out axons that correctly find their way to their target tissue. One model of how a specific group of cells can obtain their identity was proposed by Lewis Wolpert (Wolpert, 1969,1996). His so-called 'French flag' model proposed that morphogens instruct positional information to cells by forming a long-range gradient along a defined axis. Depending on distinct threshold concentrations, the morphogen activates or inhibits downstream genes, such as transcription factors. The combinatorial expression of transcription factors leads to the differentiation of specific cell types along the axis. By this mechanism, a previously unpatterned tissue can for example be instructed to take up three different identities with blue, white, and red cells along a left to right axis, forming a French flag pattern.

In order to qualify as a morphogen, a molecule has to: 1) act directly on downstream genes in the signaling pathway by activating or inhibiting these, 2) function over distance as a long-range factor, and 3) form a gradient along a defined axis. Morphogens were found to pattern many tissues in the developing body and I will later describe how morphogens are involved in several steps during vertebrate eye development.

Once the nervous system has been patterned correctly, and the right neuronal cell types have differentiated, these neurons send out axons on their journey towards their target tissue. Growing axons form a structure called a growth cone at their distal tip, which senses guidance information from the environment. These axon guidance cues can

either act in short or long range and can be attractive or repulsive in nature. Axons encounter several cues along their way, which act as intermediate guideposts to direct growth cones on the correct path towards their target tissue.

One of the miracles of the formation of a complex vertebrate nervous system is how only a handful of families of molecules can generate a network of such immense complexity as the vertebrate nervous system. More recently, it has become apparent that a single molecule can act both in tissue patterning and as axon guidance cue depending on the cellular and developmental context. One such versatile molecule, Sonic hedgehog (Shh), has been studied in the dissertation work presented here. Shh had long been known as morphogen involved in tissue patterning, but more recent research uncovered a second role as a direct axon guidance molecule. In the present work, I addressed the question of whether Shh acts by patterning the retina and optic stalk or by guiding retinal ganglion cell (RGC) axons directly to ensure correct pathfinding of these axons on their journey from the eye to the brain. Specifically, I concentrated on the first pathfinding decisions inside the retina, when RGC axons project towards the center of the retina, the optic disc, where they turn and exit the eye through the optic nerve.

I will first introduce vertebrate ocular development in general, using the zebrafish eye as an example. I will then give an introduction to Hedgehog (Hh) signaling, the molecular pathway I focused on during my dissertation work. I will discuss several steps of vertebrate eye development and highlight the role of Hh signaling for each one. I will differentiate between the roles of Shh in tissue patterning and as an axon guidance molecule and show how previous research found evidence for both in spinal cord and retinal axon pathfinding. I will then introduce the role of chemokine signaling in axon

guidance, where I found a genetic interplay between the Hh and chemokine pathways for the guidance of RGC axons out of the eye in zebrafish. Additionally, I will address why zebrafish is a great model system to study intraretinal axon guidance, and I will finally give a summary of the research conducted in the study discussed here.

Vertebrate eye development

Eye development has been intensively studied in mouse and chicken (reviewed by Chow and Lang, 2001), while the same processes are just beginning to be better understood in zebrafish (Li et al., 2000; Kwan et al., unpublished data). The general aspects of eye formation are very similar between amniotes and zebrafish and I will give a brief description of eye development, keeping zebrafish specific development in mind.

The first morphological sign of eye formation in vertebrates is the bilateral evagination of the diencephalon, forming the optic primordia. Evagination continues and an optic vesicle forms on either side of the brain. These vesicles extend distally towards the overlying ectoderm (E). At this stage, the optic vesicle (OV) can already be subdivided into several structures: the neural retina (NR) develops underlying the ectoderm. Tissue that will later develop into retinal pigment epithelium (RPE) comprises the medial part of the vesicle. The proximal elongation of the NR forms the dorsal optic stalk (OS) and the OS connects to the forebrain (F) (Figure 1.1A).

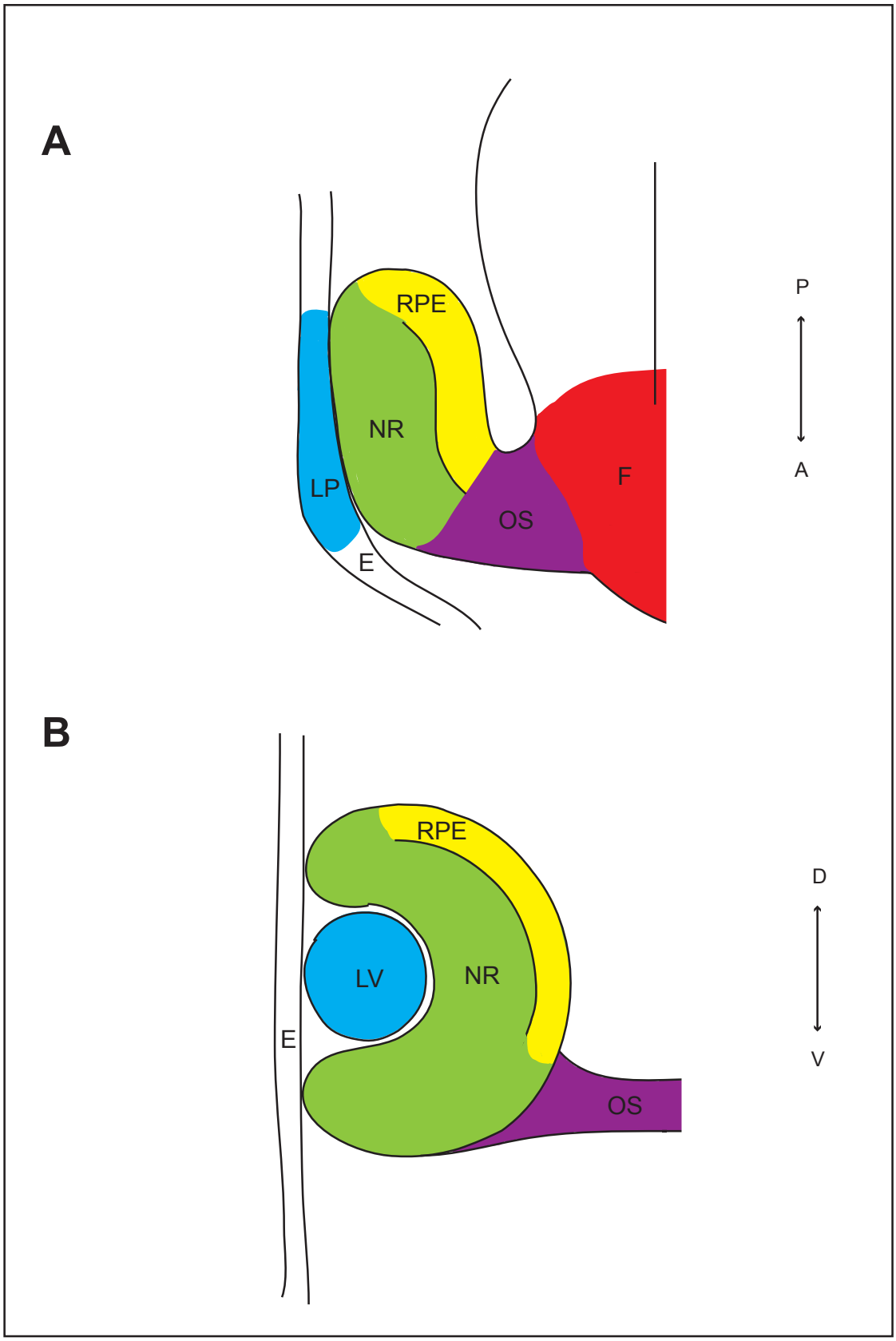
During early eye development, the facial surface ectoderm (E) covers the OV before the developing eye can even be discerned. As the OV grows it pushes outwards in contact with the ectoderm. As eye development progresses, the overlying ectoderm

Figure 1.1: Vertebrate eye development (with emphasis on zebrafish)

A) Formation of the optic vesicle through distal evagination. Presumptive neural retina (NR) comes into close contact with the lens placode (LP). The LP forms as a thickening of the surface ectoderm (E). The medial part of the optic vesicle is patterned into future retinal pigmented epithelium (RPE), while the proximal elongation of the vesicle will form the optic stalk (OS). Optic stalk tissue connects to the forebrain (F) medially. In zebrafish, the stage shown corresponds to about 16 hours of development.

B) Invagination of the optic vesicle and formation of the lens vesicle (LV) from the LP. Establishment of the two-layered eye structure with NR in the thicker inner layer and the RPE in the thin outer layer. The OS tissue forms a narrow channel through which epithelial cells can migrate into and retinal axons out of the eye. In zebrafish, the stage shown corresponds to about 24 hours of development.

blue, lens placode (LP), lens vesicle (LV)
green, neural retina (NR)
yellow, retinal pigmented epithelium (RPE)
purple, optic stalk (OS)
red; forebrain (F)


A
RPE
NR
LP
OS
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thickens and forms the lens placode (LP) (Figure 1.1A). Placode internalization then happens concurrently with OV invagination and these two processes lead to the formation of the lens and the bilayered optic cup. While the inner layer is formed by the NR, the outer layer consists of the RPE (Figure 1.1B). During invagination a groove forms along the ventral most regions of the optic vesicle and optic stalk in a proximodistal direction. This groove, called the optic fissure, together with the optic stalk provides a channel for the migration of endothelial cells into the eye and the growth of axons projecting towards the brain.

After basic eye formation, several neuronal cell types start to differentiate in the NR (Stenkamp, 2007). The first cells to be born are RGCs, which form the innermost neuronal cell type in the retina. RGCs are the only cell type extending axons that form connections with target cells in the brain, especially the superior colliculus in mammals or optic tectum in nonmammalian vertebrates. Along with these tectal connections, RGCs also synapse with nine nontectal targets. Within the retina, RGC dendrites form synapses with amacrine and bipolar cells in the inner plexiform layer. In the outer plexiform layer, bipolar and horizontal cells form connections with rod and cone photoreceptor cells. These photoreceptors make up the outermost layer of the NR and are the main photoactive cell type in the eye (Figure 1.2). The phototransduction pathway transforms light, which has travelled through the retina to stimulate photoreceptors in the outer retina, into electrical synaptic signals. These signals in turn trigger changes in RGC electrical responses in order to transport the visual signal to the brain as RGC action potentials.

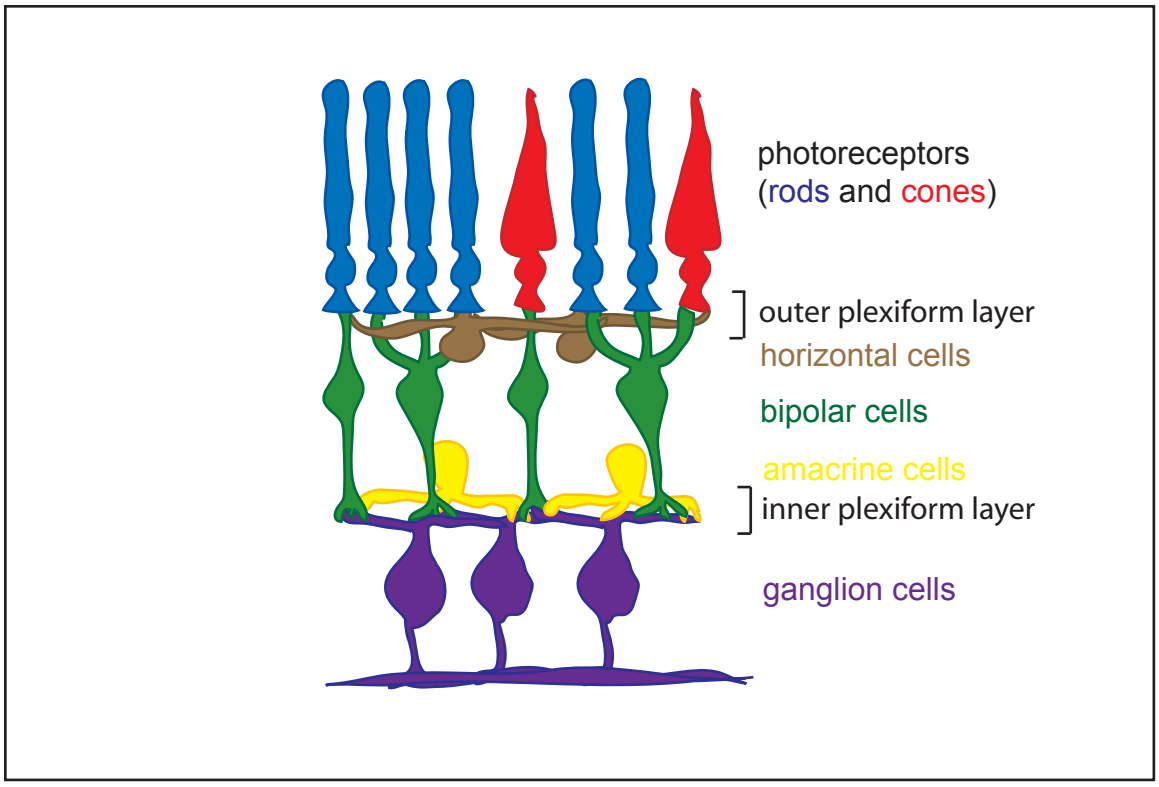


Figure 1.2: Retinal layering

The mature retina is layered into three nuclear layers interspersed by two plexiform layers. The most central layer (closest to the lens) consists of retinal ganglion cells (*purple*). The inner nuclear layer contains cell bodies from amacrine (*yellow*), bipolar (*green*), and horizontal (*brown*) cells. The outer nuclear layer is made from rod and cone photoreceptors (*blue and red*). The inner plexiform layer is formed by synaptic connections between ganglion cells and cells residing in the inner nuclear layer. The outer plexiform layer contains synapses formed by cells from the inner nuclear layer and photoreceptors.

The Hh signaling pathway

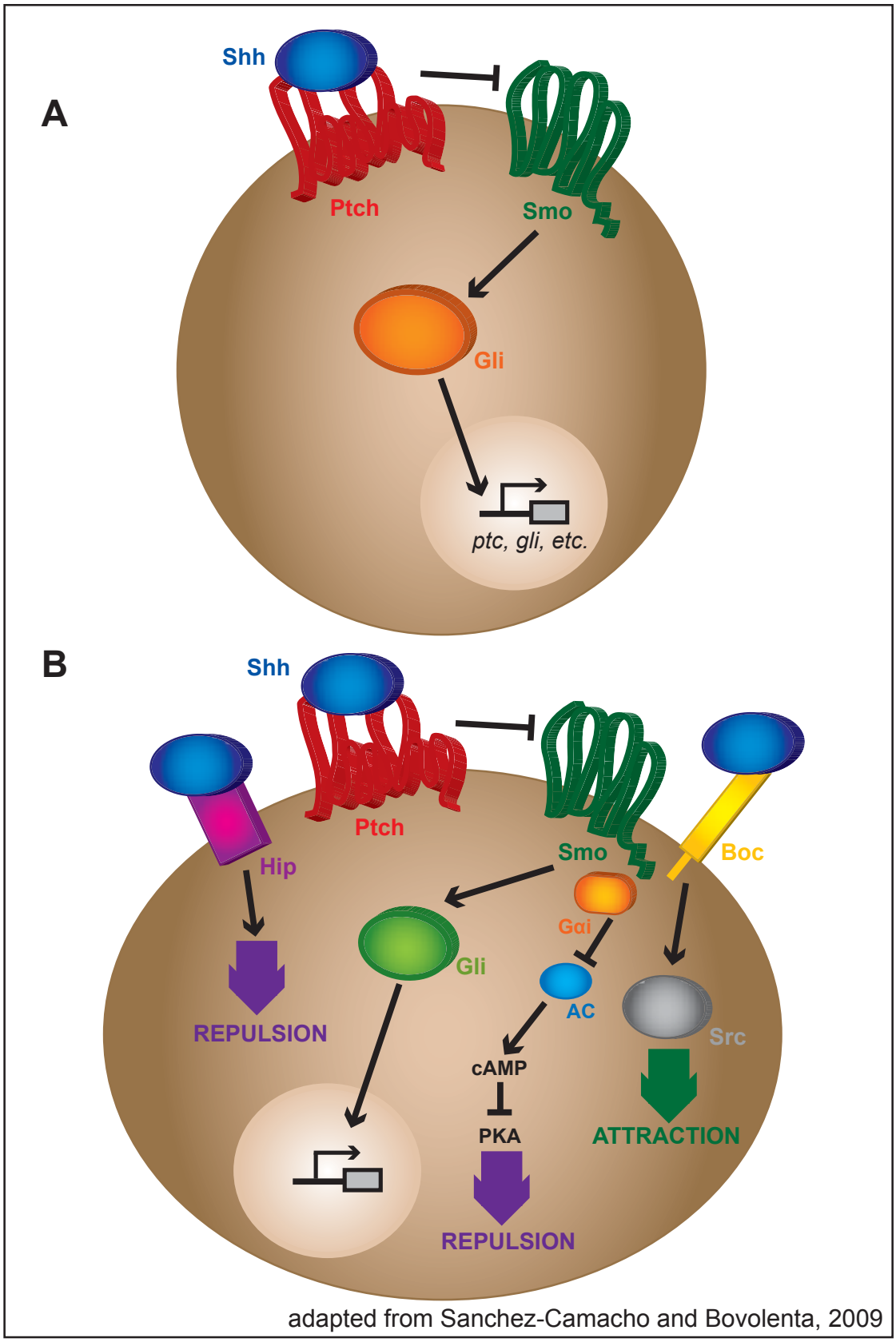
While several molecular signaling pathways have been implicated at different steps of vertebrate ocular development, during my dissertation I focused on the role of the Hh pathway. In the following sections, I will first introduce Hh signaling and then highlight the role of this pathway in eye field separation and the formation of the proximodistal and dorsoventral axes in the developing eye.

The Hh signaling pathway was first discovered in a screen for embryonic mutants affecting segment number and polarity in Drosophila (Nüsslein-Volhard and Wieshaus, 1980). This work later earned the authors the Nobel Prize in medicine and physiology in 1995. More than ten years after the description of the *hh* gene in fly, three vertebrate homologues of fly *hh* were described in mouse (Echelard et al., 1993). This study implicated one of these genes, *sonic hedgehog* (*shh*), in nervous system and limb polarity. Shh is prominently expressed in the notochord and spinal cord and acts as a ligand that binds its receptor Patched (Ptch). The receptor Ptch inhibits a second transmembrane protein, Smoothened (Smo), a G-protein coupled receptor, in the absence of Shh. Upon binding of Shh, Ptch releases this inhibition and Smo activates downstream signaling. In the pathway known as the canonical Hh signaling pathway, Smo activates Gli transcription factors, which induce Hh target gene transcription in the nucleus (reviewed in Sánchez-Camacho and Bovolenta, 2009) (Figure 1.3A). Many genes are regulated by this pathway, including members of the Hh signaling pathway itself, such as *ptch* and *gli*. Therefore, these Hh pathway genes can be used as readouts for pathway activity.

Figure 1.3: Hh signaling pathways

A) The canonical Hh signaling pathway acts in tissue patterning. The ligand Shh binds the receptor Ptch. Upon binding, Ptch releases its inhibition of Smo, a G-protein coupled receptor. Smo signals through Gli transcription factors to activate Hh target gene transcription in the nucleus.

B) Several noncanonical pathways have been proposed to be activated by the role of Shh as an axon guidance molecule. Most pathways still signal through Ptch and Smo but do not necessarily activate Gli transcription factors. Several transcription independent pathways have been proposed. For example, binding of Shh to Boc can activate Src kinases and induce an attractive response in the growth cone. Repulsive responses, on the other hand, have been proposed through binding of Shh to Hip or repression of adenylyl cyclase (AC) through Gαi signaling.



adapted from Sanchez-Camacho and Bovolenta, 2009

The canonical pathway, known for its role in tissue patterning, depends on transcriptional regulation of downstream genes for its effect. Hh signaling has also been implicated in direct axon guidance and several noncanonical Hh pathways were proposed to act independently of target gene transcription to ensure fast guidance responses at the growth cone (reviewed in Sánchez-Camacho and Bovolenta, 2009). In chicken, exposure to Shh resulted in reduced levels of intracellular cAMP and induction of axon repulsion (Trousse et al., 2001). Using primary rat neuron culture, it was suggested that Shh acts through Src kinases for attractive guidance of commissural neurons (Yam et al., 2009). In addition, Hh interacting protein (Hip) was implicated in the repulsive effect of Shh binding on postcrossing commissural neurons in chicken (Bourikas et al., 2005), while the binding of Shh to Boc was proposed to induce an attractive effect of Hh signaling on growth cones of rat commissural neurons (Okada et al., 2006) (Figure 1.3B).

Hedgehog signaling in eye field separation

Even before the two optic primordia evaginate and are morphologically visible, the eye fields are set up by specific gene expression patterns at the anterior midline. Shh is strongly expressed in the embryonic midline throughout brain development. Inhibition of the Hh signaling pathway was found to lead to failure of eye field separation, resulting in cyclopia in organisms ranging from zebrafish, to chicken, mouse and humans (Barresi et al., 2000; Belloni et al., 1996; Chiang et al., 1996; Hu and Helms, 1999; Pera and Kessel, 1997; Roessler et al., 1996).

Additional studies started to reveal the molecular mechanisms involved in eye field separation and the regulation of *shh* expression at the midline. In zebrafish, the two

separate eye fields are derived from a single medial field of retinal precursor cells, which express *zic family member 1* (*zic1,* a member of C2H2-type zinc finger proteins) at the end of gastrulation (Varga et al., 1999). The movement of *foxb1.2* (a forkhead box trancription factor) positive diencephalic precursor cells anteriorly along the midline during late gastrulation and early segmentation stages leads to the separation of the two eye fields and the formation of the ventral diencephalon (Varga et al., 1999). In zebrafish *cyclops* mutants, which carry a mutation in the *nodal-related 2* (*ndr2,* a member of the TGF-beta superfamily) gene, the diencephalic precursor cells express *zic1* instead of *foxb1.2* and fail to move anteriorly. As a result, the eye fields fail to separate and the mutant embryos form a single cyclopic eye at the midline, hence the name *cyclops* (Varga et al., 1999). *Ndr2* expression is required for the induction of the ventral neural tube and the expression of *shh* at the ventral midline (Muller et al., 2000; Tian et al., 2003), suggesting that the cyclopic phenotype in *nodal* mutants can at least in part be explained by the loss of Hh signaling at the midline.

Retina vs. optic stalk – Hedgehog signaling in patterning of the proximodistal axis

After separation of the two eye fields, the developing optic tissue has to be patterned into future retinal and optic stalk tissue along the proximodistal axis. Again, Hh signaling has been implicated in this process. Here, the gradient of Shh emanating from the midline sets up the specific expression domains of two paired box transcription factors, *pax2* and *pax6,* along the proximodistal axis (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). Proximal high Shh levels induce the expression of *pax2*

transcription factor and the formation of the future optic stalk, while distal low Shh leads to the activation of Pax6 and the development of this tissue into the optic vesicle (Figure 1.4). The loss of Shh leads to an expansion of Pax6 expression in expense of Pax2, which results in the failure of optic stalk formation. Shh misexpression in the optic vesicle, on the other hand, promotes the formation of proximal cell fates and an enlargement of the optic stalk (Ekker et al., 1995).

Hedgehog signaling in dorsoventral eye patterning

After the specification of the optic cup, the eye is also patterned in the dorsoventral (DV) axis. One more time, Hh signaling was proposed to take part in determining the DV axis in the developing eye. Bone morphogenetic proteins (Bmps) from the dorsal eye induce the expression of dorsal cell fate (Figure 1.4). Shh secreted by ventral eye tissue, on the other hand was given the role as a player in inducing ventral cell fate. By restricting each other's expression to the opposite side of the eye, Shh and Bmp induce ventrally and dorsally expressed transcription factors, respectively (Zhao et al., 2010; Sasagawa et al., 2002; Behesti et al., 2006) (Figure 1.4). The *ventral anterior homeobox 2* (*vax2*) expression in the ventral retina is induced by Shh and it was shown in both mouse and chicken that *vax2* expression is required for the induction of ventral retinal cell fate (Schulte et al., 1999; Barbieri et al., 1999, 2002; Zhang and Yang, 2001; Mui et al., 2002), while the induction of *T-box 5* (*tbx5*) transcription factor by Bmp in the dorsal eye is necessary for dorsal retinal cell fate (Koshiba-Takeuchi et al., 2000; Behesti et al., 2006). Additionally, a more recent study in zebrafish showed that the diffusible factor Gdf6a (Growth differentiation factor 6a, a member of the TGF-beta superfamily) is

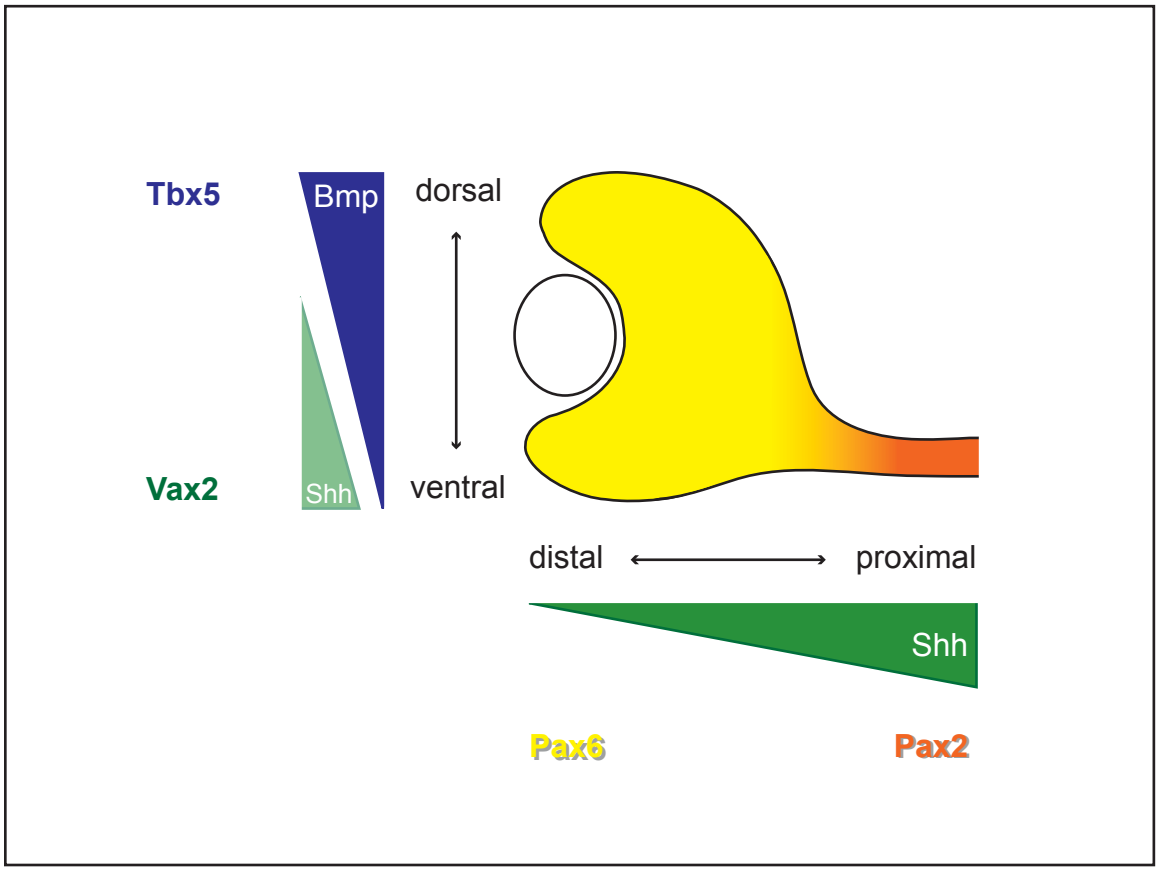


Figure 1.4: Proximodistal and dorsoventral eye patterning

A gradient of Shh secreted by the midline patterns ocular tissue in the proximodistal axis. A proximal high levels of Shh induce Pax2 expression and optic stalk formation, while distal low levels induce Pax6 expression and retinal tissue identity.

The dorsoventral axis of the retina is being set up by dorsal high levels of Bmp, which induce Tbx5 expression and dorsal retinal cell fate. It was proposed that the dorsalizing effect of Bmp is counteracted by a Shh gradient from the ventral retina, which induces Vax2 expression and ventral cell fate. But at this point it is not clear whether the role of Shh in determining ventral cell fate in the retina is a direct effect or whether this is a secondary effect to its role in setting up the proximodistal axis (faded Shh gradient).

required for the induction of dorsal retinal cell fate and is sufficient to induce the expression of the dorsal markers *bmp4*, *tbx5*, *tbx2b* and *ephrin-b2* (a receptor protein-tyrosine kinase) in the dorsal retina and the suppression of *vax2* in the ventral eye (Grosse and Baier, 2009).

Whether the ventralizing role of Shh in the retina is a direct effect of a dorsoventral Shh gradient, or whether the role in determining the dorsoventral retinal axis is secondary to the role of Shh in setting up the proximodistal axis is not clear at this point. At least in Xenopus it was shown that inhibition of the Hh signaling pathway using cyclopamine treatment did affect Pax2 and Pax6 expression domains and led to severe morphological defects in the ventral eye, but had no effect on Vax2 expression in the ventral retina (Perron et al., 2003), supporting the hypothesis that Hh signaling is primarily required for proximodistal ocular patterning.

Hedgehog signaling in retinal cell differentiation

After optic tissue patterning, retinal cell types differentiate in a temporally and spatially controlled manner. It was proposed that signals emanating from the optic stalk induce neurogenesis in the zebrafish retina (Masai et al., 2000). The first neuronal cells to differentiate are RGCs, which are located in the inner most layer of the retina. Shortly after RGC differentiation begins, cone photoreceptors, horizontal and amacrine cells start to differentiate, followed by rod photoreceptor cells, bipolar and Müller glia cells. The differentiation of all retinal cell types results in the precisely organized retina with three nuclear layers, which are interspersed with two plexiform layers, where synaptic connections are formed. In zebrafish, RGC differentiation nucleates in a ventronasal

patch and progresses in a wave-like manner circumferentially around the central retina until about 40 hours post fertilization (hpf), at which point more peripheral RGCs start to differentiate (Hu and Easter, 1999). The wave of RGC progression in zebrafish waspropposed to require the expression of Shh in RGCs (Neumann and Nüsslein-Volhard, 2000). A different study proposed that retina intrinsic signals are not necessary for RGC differentiation in zebrafish and proposed, on the other hand, that Shh from the midline is important for the spread of the neurogenic wave (Kay et al., 2001). This finding is in concert with a signal emanating from the optic stalk proposed earlier (Masai et al., 2000). Additionally, it was shown that RGC differentiation in zebrafish depends on the cell autonomous expression of the basic helix-loop-helix (bHLH) proneural transcription factor *atonal homolog5 (ath5;* later renamed *atoh7*) in precursor cells, since zebrafish mutant for *ath5* do not differentiate RGCs but instead develop an excess of amacrine, bipolar, and Müller glia cells (Kay et al., 2001). In addition to midline Shh and the wave of Shh in RGCs, two more waves of Shh expression occur in the zebrafish eye. A second wave of Shh expression was detected in amacrine cells (Shkumatava et al., 2004). This amacrine wave spreads independently but almost simultaneously with the wave of Shh expression in RGCs. Shh secretion from amacrine cells acts as a short-range signal to regulate differentiation of all other cell types in the inner and outer nuclear layer of the zebrafish retina. A third wave spreads through the RPE layer and is associated with the differentiation of photoreceptor cells in the outer nuclear layer of the retina (Stenkamp et al., 2000).

In contrast to amniotes where retinal cell proliferation halts after the entire retina has been filled with differentiated neurons, in nonamniotes such as zebrafish and

Xenopus, the retina grows throughout life by addition of new neuronal cells generated in the ciliary marginal zone (CMZ) (Straznicky and Gaze, 1971; Johns, 1977).

Forming retinotectal connections – retinal axon guidance

In order to convey visual information from the retina to higher brain regions, RGC axons need to form synaptic connections with their target cells in the optic tectum. Before reaching the tectum, RGC axons have to make multiple pathfinding decisions along their way. After cell differentiation, RGCs extend axons, which project towards the optic disc in the center of the retina. After reaching the disc, axons have to turn and exit the eye through the optic nerve (Figure 1.5). Then, traveling along the optic nerve, axons reach the chiasm. Here, all axons cross the midline and project to the contralateral side in zebrafish, while in mouse and humans, only nasal RGC axons cross, and lateral axons project to the ipsilateral side. After this pathfinding decision at the midline, axons project along the optic tract to their final target, the optic tectum.

Molecular pathways involved in intraretinal axon pathfinding

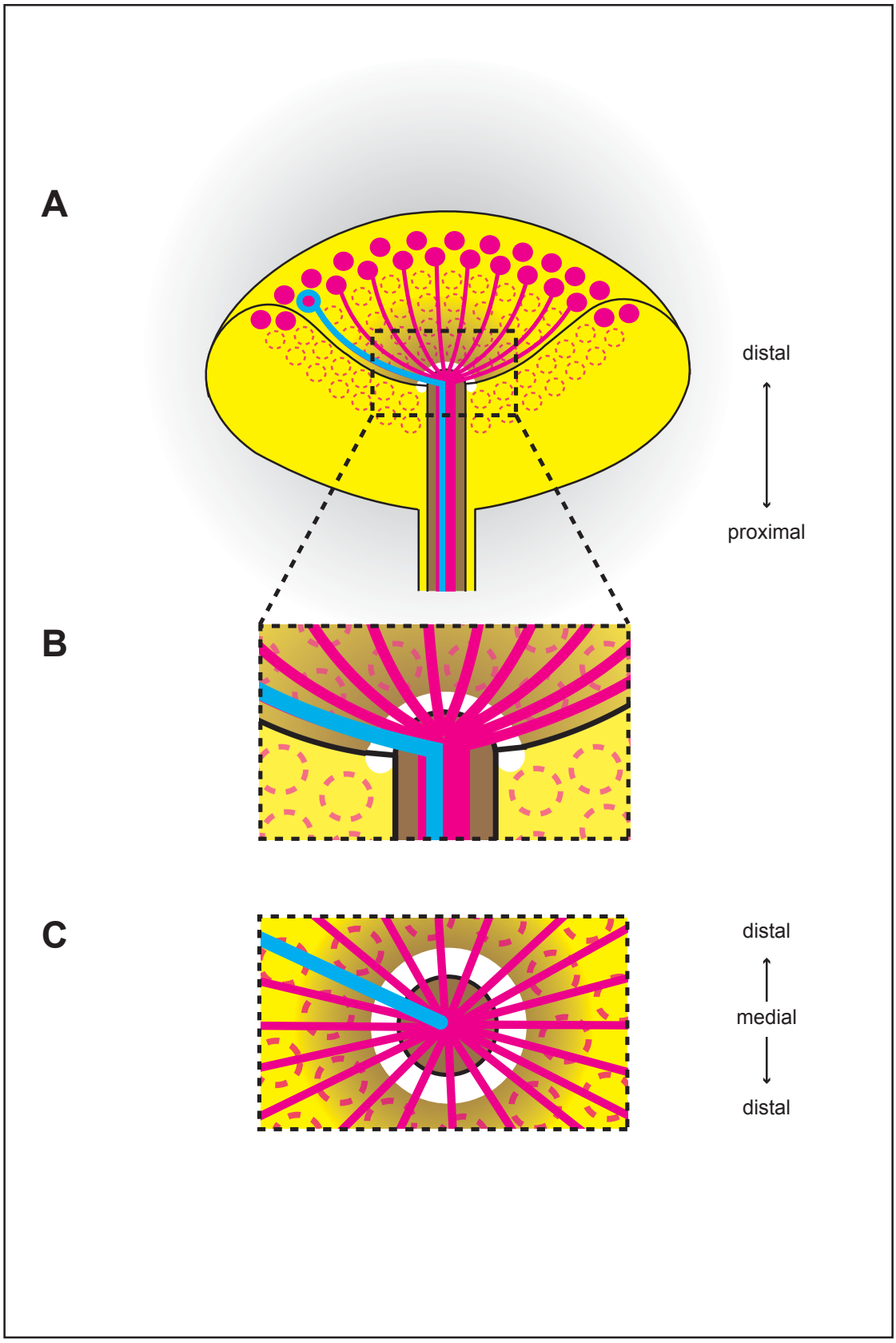
My dissertation project focused on the guidance of RGC axons within the retina itself. While a number of axon guidance molecules have been implicated in guiding RGC axons towards the optic tectum, I will here address only molecules involved in the guidance of RGC axons within the eye. Some molecules required for correct intraretinal axon pathfinding have been discovered through phenotypic analysis of mutant animals.

Figure 1.5: Intraretinal axon pathfinding

A) Schematic showing hemisection of retina (yellow). RGCs (*pink*) differentiate in the inner most layer of the retina. They send out axons that project towards the optic disc (*white*) in the center of the retina. Here, RGC axons turn and exit the eye through the optic nerve (*brown*). Path of a single axon is highlighted in *blue*.

B) Blow-up of optic disc region. Highlighted axon (*blue*) turns (*arrow*) after reaching the optic disc (*white*).

C) Schematic of optic disc region as seen through the lens. Axons converge at the optic disc and exit the eye through the optic nerve.


A
distal
proximal
B
C
distal
medial
distal

The findings from several different species are summarized here to give an overview of the molecular pathways known to be involved in intraretinal axon pathfinding to date. An early study analyzing intraretinal axon pathfinding reported that chondroitin sulfate proteoglycans (CSPGs), expressed in a peripheral high and central low concentration, guide RGC axons in a repulsive manner towards the optic disc in rats (Snow et al., 1991). Protein localization of CSPGs seemed to shift peripherally, in concert with RGC differentiation, always with high levels of CSPGs at the outer edge of the RGC layer. This study in rats therefore suggested that, while laminin promotes axon elongation, CSPGs direct axon outgrowth towards the optic disc by repulsion. Unfortunately, this result could not be replicated in other species.

Another study showed in goldfish that cell adhesion molecules, such as Neurolin and L1, are necessary to guide dorsal RGC axons in an orderly manner towards the optic disc (Ott et al., 1998). After injection of Neurolin Fab fragments RGC axons defasciculated, grew in circles, and failed to reach the optic disc.

The axon guidance molecule Netrin-1 is expressed in neuroepithelial cells at the optic disc, while its receptor, DCC, is expressed in RGCs (Höpker et al., 1999; Serafini et al., 1996; Keino-Masu et al., 1996). In mice deficient for Netrin-1 or DCC, it was shown that axon pathfinding towards the disc was unaffected, while axons failed to exit the eye through the optic stalk (Deiner et al., 1997). These findings suggest a role for Netrin-1 in guiding RGC axons locally at the optic disc in mice.

In addition, EphB receptors were proposed to act as inhibitory guidance cues for the growth of dorsal RGC axons towards the optic disc in mice (Birgbauer et al., 2000; 2001). In EphB2 and B3 double mutant mice, dorsal intraretinal axon pathfinding errors

were characterized by failure to exit through the optic disc and instead overshooting the disc towards the ventral retina. While correct mapping of RGC axons in the tectum is dependent on EphB kinase activity, the role of EphB receptors in intraretinal axon pathfinding seemed to be kinase-independent. The ventral high-dorsal low gradient of EphB2 was proposed to have an inhibitory effect on RGC outgrowth and therefore prevent dorsal axons from entering the ventral retina.

Later, Slit proteins, signaling through the Robo2 receptor, were proposed to help restrict ventral RGC axons to the optic fiber layer on their way to the optic disc in mice and control initial directionality of axon outgrowth in dorsal RGCs (Thompson et al., 2006, 2009). While axon outgrowth within the retina was disturbed in these animals, exit from the eye did not seem to be affected.

Recently, the role of heparan sulfate (HS) in intraretinal axon pathfinding was shown in mice by ablation of the Ext-1 enzyme (Ogata-Iwao et al., 2011). This study proposed that HS is essential for axon pathfinding towards the optic disc by modulating Netrin-1 and Slit-mediated axon guidance.

In zebrafish, the chemokine ligand Cxcl12a (formerly known as stromal cell derived factor 1a, Sdf1a), is expressed at the optic disc, while its receptor Cxcr4b is strongly expressed in RGCs (Li et al., 2005). It was shown that loss of chemokine signaling leads to intraretinal axon pathfinding errors. Using Cxcl12 misexpression within the retina, it was proposed that Cxcl12 has an attractive effect on RGC axons inside the eye (Li et al., 2005).

Last but most relevant for my study, mutants for *shh* in both mice and zebrafish show prominent intraretinal axon pathfinding errors where axons fail to exit the eye and

instead project within the retina (Dakubo et al., 2003; Sánchez-Camacho and Bovolenta, 2008; Schauerte et al., 1998). How the Hh signaling pathway regulates intraretinal axon pathfinding in these organisms is not clear. First, pathfinding errors could occur because of defects in ocular tissue patterning. Second, the phenotype could be due the loss of Shh as a direct axon guidance cue.

For my dissertation, I analyzed the role of Hh in intraretinal axon pathfinding using a single organism, zebrafish, and multiple avenues of attack, to clarify these possiblities.

What is the role of Sonic hedgehog in intraretinal axon pathfinding in zebrafish?

As mentioned before, zebrafish mutants for *shh* exhibit strong intraretinal axon pathfinding errors (Schauerte et al., 1998). Instead of leaving the eye through the optic nerve, RGC axons fail to exit and project posteriorly, and sometimes anteriorly, within the eye, and become trapped.

It was shown and we have confirmed that in the zebrafish eye Hh pathway components are expressed both at the embryonic midline and later in the RGC layer (Krauss et al., 1993; Neumann and Nüsslein-Volhard, 2000) (Chapter 3). Due to these expression patterns, we can imagine two likely mechanisms of how Hh signaling regulates the pathfinding of RGC axons out of the eye, which are both supported by previous research in mice (Dakubo et al., 2003; Sánchez-Camacho and Bovolenta, 2008). First, Shh could act as a direct axon guidance molecule, directing RGC axons on their journey out of the eye by activating Hh receptors in RGC growth cones. Second, Hh

signaling could regulate RGC axon pathfinding indirectly, acting through tissue patterning and the regulation of the expression of other direct guidance molecules in the eye.

I will give evidence for both roles, starting with findings for Hh signaling involved in tissue patterning and then evidence for a role in direct axon guidance.

Sonic hedgehog in tissue patterning

The role of Shh as an important morphogen was first discovered by studies directed towards understanding how different types of neurons are produced at distinct positions within the spinal cord. The expression of Shh in the notochord and ventral spinal cord made this protein a prime candidate for a morphogen setting up a gradient in the spinal cord. The graded DV localization of Shh protein was confirmed in mouse, rat and chick spinal cord (Roelink et al., 1995) and further studies suggested a role for Shh in regulating the induction of distinct neuronal cell types (Martí et al., 1995; Ericson et al., 1996, 1997; Chiang et al., 1996). Thus Shh fulfills all three qualities a molecule has to meet to be called a morphogen: forming a gradient, acting as long-range factor, and acting directly on downstream genes.

While the role of Shh as morphogen involved in patterning of the spinal cord is undoubtedly one of its more prominent ones, Hh signaling is also known to pattern vertebrate limbs by regulating digit formation (Chang et al., 1994; Yang et al., 1997), and most importantly for my studies, Hh signaling was implicated in patterning of ocular tissue at several steps during eye development (reviewed in Martí and Bovolenta, 2002). The loss of *shh* during early embryonic development leads to failure of eye field

separation and to cyclopia (Chiang et al., 1996). In addition, Hh signaling was shown to be involved in the establishment of the proximodistal axis during eye development both in zebrafish and Xenopus (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). Injection of *shh* RNA in zebrafish led to failure of the separation of the eye primordium from the diencephalon and these embryos showed an increase in Pax2 expression in the optic primordium with a reduction in the number of Pax6 expressing cells. Together, these phenotypes were proposed to be the result of a hypertrophied optic stalk and a decrease of neural retina differentiation (Ekker et al., 1995; Macdonald et al., 1995). Similarly, in Xenopus it was shown that pathway activation by overexpression of a dominant-negative form of PKA or *shh* led to the transformation of the ventral retina into optic stalk tissue. This phenotype was again accompanied by an expansion of the *Pax2* domain and a decrease of *Pax6* expression (Perron et al., 2003). Additionally, further studies in zebrafish proposed a model where Nodal, upstream of Hh signaling, is necessary for the formation of midline structures. Loss of Nodal leads to loss of Hh pathway gene expression at the midline. Hh signaling normally induces *pax2* and *vax* gene expression in the optic stalk region and the failure to induce these transcription factors after loss of Hh signaling leads to failure of optic stalk formation and fissure closure, resulting in coloboma in zebrafish (Take-uchi et al., 2003).

In mouse, an additional mechanism was proposed for how Hh signaling regulates optic stalk cell formation (Wallace and Raff, 1999; Dakubo et al., 2003, 2008). These studies showed that astrocyte proliferation in the optic nerve was stimulated by Shh. Dissection of the eye resulted in loss of *ptch* expression in the optic nerve, suggesting that the source of Shh responsible for astrocyte proliferation comes most likely from RGC

axons as they leave the eye (Wallace and Raff, 1999). This was later confirmed by conditional ablation of *shh* in RGCs, which resulted in loss of optic disc astrocyte precursor cells. These mice exhibited intraretinal axon guidance errors similar to the phenotype observed in *netrin-1* mutants (Deiner et al., 1997). Analysis of *netrin-1* levels at the optic disc revealed a complete loss of expression in the conditional *shh* mutants, making it likely that the intraretinal axon guidance errors were the result of loss of *netrin-1* expression in optic disc astrocytes (Dakubo et al., 2003).

In summary, these studies implicate Hh signaling in patterning of the proximodistal axis into optic stalk and retinal tissue in zebrafish and Xenopus and also in formation of optic stalk astrocytes in mice. While axis formation was proposed to depend on Shh secretion from the midline, optic stalk astrocyte proliferation was suggested to be regulated by Shh from RGC axons. These studies highlight a possible species-specific difference in the source and mechanism of the involvement of Hh signaling in ocular tissue patterning and subsequent intraretinal axon pathfinding.

Sonic hedgehog as axon guidance molecule

In contrast to its role in tissue patterning, more recently it was proposed that Shh acts directly on growth cones to regulate their guidance. While canonical Hh signaling involved in tissue patterning and cell specification acts through Gli transcription factors to activate Hh target gene transcription, the role of Hh signaling in axon guidance is thought to be independent of gene transcription, and several noncanonical pathways have been proposed (reviewed in Sánchez-Camacho and Bovolenta, 2009).

Initial evidence for Shh functioning as a direct axon guidance molecule came from *in vitro* and *in vivo* studies in chicken showing that Shh acts as a negative regulator of growth cone movement (Trousse et al., 2001). This study proposed that the localized downregulation of Shh at the chiasm defines a constrained pathway, which serves to guide RGC axon outgrowth across the midline. Later, additional *in vitro* experiments using chick retinal explants proposed that Shh has a dual effect on RGC axon outgrowth depending on its concentration (Kolpak, 2005). This study showed that low Shh concentrations have a positive effect on axon outgrowth, while high concentrations repress axon outgrowth. Using intraocular injections of cyclopamine to inhibit Hh signaling at the beginning of RGC differentiation, this study also suggested that Hh signaling acts in chicken directly on axon guidance and not through patterning of the eye. The same group later proposed that this negative effect of Shh in the chick retina is mediated through macropinocytosis (Kolpak et al., 2009). Additionally, studies in mouse proposed that Shh from RGCs mediates RGC axon growth in a cell autonomous manner out of the eye and Shh from the midline guides axons across the chiasm (Sánchez-Camacho and Bovolenta, 2008). More recently, it was reported that the segregation of ipsi-and contralateral RGC projections at the chiasm in mice requires the expression of the Shh receptor Boc in ipsilateral axons (Fabre et al., 2010). Studies in Xenopus indicate that Shh not only regulates guidance of RGC axons at the chiasm but also postcrossing axon pathfinding along the optic tract (Gordon et al., 2010).

In addition to retinal axon guidance, Shh has been implicated in commissural axon guidance. Here, a number of alternate downstream signaling pathways have been proposed. First, it was shown that Shh acts as a chemoattractant that collaborates with

Netrin-1 at the midline in the embryonic mouse spinal cord (Charron et al., 2003). Later it was proposed that Shh mediates this effect through the noncanonical receptor Boc, which is expressed in commissural axons (Okada et al., 2006). In chicken it was shown that Shh guides commissural axons along the longitudinal axis and that this effect is mediated by the interaction of Shh with Hip (Bourikas et al., 2005). Later, the same group proposed that Shh guides these postcrossing axons directly and indirectly by shaping a gradient of Wnt activity (Domanitskaya et al., 2010). Additionally, Shh was proposed to act as a switch to turn on the repulsive activities of Semaphorins at the rat embryonic midline (Parra and Zou, 2010). To reveal further downstream signaling components, an *in vitro* assay was developed to record axon outgrowth responses to defined gradients of chemical cues (Yam et al., 2009). This study showed that commissural axons turn towards increasing concentrations of Shh. This response was proposed to be independent of transcription but is instead signaling through Src family kinases, supporting the existence of non-canonical Hh signaling pathways in axon guidance.

In conclusion, while Hh signaling was shown to be involved in patterning of ocular tissue through an indirect role in intraretinal axon pathfinding by regulating the expression of downstream genes, a growing body of work implicates Hh signaling also in direct guidance of RGC axons out of the eye through the role of Shh as a guidance cue. Whether this controversy is due to a difference in experimental design or reflects species and temporal differences in the role of Hh signaling during ocular development has remained unclear. Using zebrafish, I clarified the involvement of Hh signaling in intraretinal axon pathfinding in a single cell type, RGCs, and showed that here, intraretinal axon pathfinding errors are due to a role of Hh signaling in patterning of the

optic stalk and the regulation of downstream molecules, which act as direct guidance cues to guide RGC axons out of the eye.

Chemokine signaling in axon guidance

Zebrafish chemokine mutants exhibit intraretinal axon guidance errors almost indistinguishable from the ones found in Hh mutants (Li et al., 2005). This phenotypic similarity prompted me to study a possible interaction between the Hh and chemokine pathways for the guidance of RGC axons out of the eye in zebrafish. Therefore, I will, at this point, give a short introduction to chemokine signaling in axon guidance.

Chemokines are a family of small cytokines expressed by cells in the immune and nervous system. Chemokines bind to G-protein coupled transmembrane receptors on target cells, called chemokine receptors. A small body of work implicated chemokine signaling in axon pathfinding. Through *in vitro* experiments using chicken and mouse neuronal cultures and *in vivo* studies in zebrafish, Cxcl12 has been proposed to act as a modulator of repulsive guidance cues such as Slits and Semaphorins (Chalasani et al., 2003; 2007). Additionally, this anti-repulsive effect was described to be mediated by a pathway, which acts through calmodulin-stimulated adenylate cyclase to elevate cAMP levels (Xu et al., 2010). On the other hand, chemokine signaling was also implicated in direct axon guidance inside the eye in zebrafish (Li et al., 2005). This study showed that ectopically expressed Cxcl12 had an attractive effect on RGC axons within the eye. *In vitro* studies using rat cerebellar granule neuron cultures, showed a repulsive turning response in axon growth cones away from a gradient of Cxcl12 (Xiang et al., 2002). The

analysis of spinal cord axonal projections in *cxcr4* mutant mice revealed pathfinding defects and hyperfasciculation (Chalasani et al., 2003).

In summary, chemokine signaling has been implicated in axon pathfinding as a modulator of other guidance molecules and as direct axon guidance cue acting either in an attractive or repulsive manner in a variety of systems. While downstream signaling through regulation of cAMP and PKA levels have been described and the interaction with and modulation of other guidance cues have been proposed, upstream mechanisms regulating *cxcl12* expression and its effectiveness as axon guidance molecule have not been proposed. During my dissertation, I revealed that the expression of the chemokine *cxcl12a*, which acts as direct axon guidance molecule, is regulated by the Hh signaling pathway.

Zebrafish retinal axon pathfinding as experimental model system

We have chosen to conduct this study using the zebrafish eye as a model system due to a number of technical advantages. First, the maintenance of zebrafish is relatively simple and cheap. Second, zebrafish eggs are externally fertilized and the ex-utero developing embryos are therefore available for experimentation starting from the one cell stage. Third, embryos are optically transparent and develop to the required stages for the work described here within only two days. Both of these aspects allow for fast and easy imaging methods necessary for this work. Fourth, morpholino (MO) oligonucleotide injections are a quick and established method for targeted downregulation of gene expression in zebrafish (Nasevicius and Ekker, 2000). I used well-established MOs for *ath5* and *twhh* for my experiments. Fifth, generation of transient and stable transgenic

zebrafish has been simplified by the generation of a multisite gateway-based construction kit integrated with transposon-based insertional strategies (Kwan et al., 2007).

Sixth, the possibility of carrying out large-scale forward genetic screens has yielded a large array of useful mutants from several different screens. Many of the mutants I used for my studies were found during a screen conducted in Tübingen, searching for mutations affecting retinotectal axon pathfinding and topographic mapping (Mullins et al., 1994; Karlstrom et al., 1996; Trowe et al., 1996). It was especially useful to be able to address questions regarding the Hh signaling pathway with null mutants for both the ligand *shh* and its receptor component *smo*. Since the zebrafish genome harbors only one *smo* gene, mutants for this gene are null for the Hh signaling pathway, apart from maternal *smo* mRNA expression in the early embryo (Varga et al., 2001). In addition to using mutants of the Hh signaling pathway, I was also fortunate to have mutants of the chemokine pathway available, both the ligand *cxcl12a* and the receptor *cxcr4b* (Valentin et al., 2007; Knaut et al., 2003).

Seventh, as well as genetic manipulations using morpholino and mutant strategies, zebrafish are also amenable to pharmacological treatments by bath application of the desired drug. This approach allows for easy temporal control of gene inhibition. For my project, pharmacological inhibition of *smo* using the small molecule inhibitor SANT75 (Yang et al., 2009) was used to determine the temporal requirement for Hh signaling in intraretinal axon pathfinding.

Finally, the external development of zebrafish allows for ease of embryological manipulations, such as cell transplants. Using retinal cell transplants, I determined the

non-cell-autonomous gene functions of components of the Hh signaling pathway. These experiments would be a real challenge using another system, such as mouse.

Research summary

My dissertation research aimed to elucidate the role of one of the master developmental molecules, Shh, in regulating the growth of RGC axons out of the zebrafish eye. While early studies revealed Shh as one of the major morphogens involved in patterning of the embryonic nervous system and specifically optic stalk and retina patterning (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003), more recent research has given it an additional role, acting in the process of axon guidance in both spinal cord and retinal neurons (Trousse et al., 2001; Kolpak et al., 2005; Sánchez-Camacho and Bovolenta, 2008; Fabre et al., 2010; Gordon et al., 2010; Charron et al., 2003; Bourikas et al., 2005; Okada et al., 2006; Domanitskaya et al., 2010; Parra and Zou, 2010; Yam et al., 2009). Interestingly, zebrafish mutant for *shha* (commonly called *sonic you*, *syu*) exhibit prominent intraretinal axon pathfinding errors. Previous research left the mechanism of how Hh signaling regulates intraretinal axon pathfinding unclear. Therefore, we set out to determine if Shh acts in patterning of zebrafish ocular tissue to regulate intraretinal axon pathfinding indirectly, or if Shh acts in direct axon guidance.

Chapter 2 of this work is a reprint of a recently published methods chapter describing genetic and labeling methods to analyze RGC axon guidance in zebrafish (Poulain et al., 2010). Personally, I revived a focal dye labeling technique for precise labeling of small bundles of RGC axons. This method is useful to visualize RGC axon projections within the retina and along the optic tract, as well as axon targeting and

dendritic arborization in the optic tectum. Focal dye labeling was used in my study to characterize the intraretinal axon pathfinding phenotype of Hh pathway mutants (Chapter 3, Figure S3.1).

The main body of work in this dissertation consists of the research done on the role of Shh in intraretinal axon pathfinding in zebrafish. Chapter 3 of this work describes the experiments done and results found through this analysis. I found that Shh acts in the process of RGC axon pathfinding in zebrafish indirectly as a morphogen required for correct patterning of the optic stalk but not directly as axon guidance cue. Additionally, this research reveals a novel interaction of the Hh and chemokine signaling pathways for intraretinal axon pathfinding in zebrafish, proposing that Shh regulates the expression of the guidance molecule *cxcl12a* at the optic disc.

Finally, Chapter 4 gives a conclusion of the results found in this study and addresses possible directions for future research aiming to further elucidate the mechanisms involved in RGC axon guidance.

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CHAPTER 2

ANALYZING RETINAL AXON GUIDANCE IN ZEBRAFISH

The following chapter is a reprint from a book chapter coauthored by Fabienne E. Poulain, John A. Gaynes, Cornelia Stacher Hörndli, Mei-Yee Law, and Chi-Bin Chien, published in *Methods in Cell Biology Volume 100, The Zebrafish: Cellular and Developmental Biology, Part A*. Reprinted with permission.

CHAPTER 1

Analyzing Retinal Axon Guidance in Zebrafish

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Abstract

How neuronal connections are established during development is one of the most fascinating questions in the field of neurobiology. The zebrafish retinotectal system offers distinct advantages for studying axon guidance in an *in vivo* context. Its accessibility and the larva's transparency not only allow its direct visualization, but

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also facilitate experimental manipulations to address the mechanisms of its development. Here we describe methods for labeling and visualizing retinal axons *in vivo*, including transient expression of DNA constructs, injection of lipophilic dyes, and time-lapse imaging. We describe in detail the available transgenic lines for marking retinal ganglion cells (RGCs); a protocol for very precise lipophilic dye labeling; and a protocol for single cell electroporation of RGCs. We then describe several approaches for perturbing the retinotectal system, including morpholino or DNA injection; localized heat shock to induce misexpression of genes; a comprehensive list of known retinotectal mutants; and a detailed protocol for RGC transplants to test cell autonomy. These methods not only provide new ways for examining how retinal axons are guided by their environment, but also can be used to study other axonal tracts in the living embryo.

I. Introduction

Axon guidance is an essential process for proper formation of neuronal connections during development. This is certainly true in the visual system, where retinal axons must interpret a large variety of signals to navigate to their brain target and establish precise and ordered connections reflecting our perception of the environment. The accessibility of the visual system not only allows its easy visualization, but also facilitates experimental manipulations to test the mechanisms of its development. Many studies have taken advantage of this accessibility to give a precise description of the visual system's anatomy and identify important factors required for its formation. In the past decade, the zebrafish retinotectal system has drawn attention for its distinct advantages. The optical transparency of zebrafish embryos allows direct visualization of retinal axons and is particularly suited for high-resolution imaging, including time-lapse analysis. Chimeric embryos with retinal neurons of different genetic backgrounds can be easily generated by cell transplants. Finally, the short generation time of zebrafish as well as the recent characterization of its genome are especially suited for genetic analysis and have allowed the generation and identification of many mutants with retinotectal defects. These properties establish zebrafish as an excellent model for studying retinal axon guidance and, more generally, for studying cell biology in an *in vivo* context, as many *in vivo* experiments not possible in other systems can be performed.

Retinal ganglion cells (RGCs) are the primary cell type in the innermost cellular layer of the retina, responsible for carrying visual information from the eye to the brain. In zebrafish, the first RGCs are born at 28 h post-fertilization (hpf) (Hu and Easter, 1999; Masai *et al*., 2005) and immediately extend axons that then must pass several landmarks (Fig. 1A). Retinal axons first grow within the retina to the optic disc, where they exit (30–32 hpf). They then join the optic nerve and elongate toward the ventral midline of the diencephalon, where nerves coming from both eyes meet to form the optic chiasm (34–36 hpf). In zebrafish and other species lacking binocular vision, all axons cross the midline. Retinal axons then

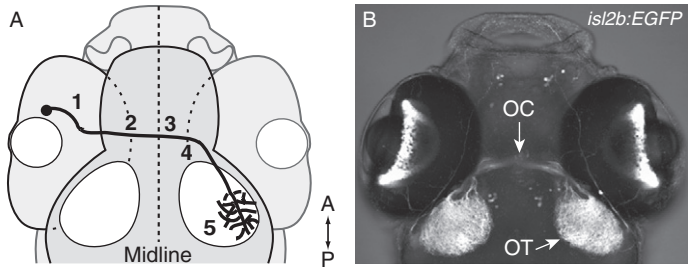

Fig. 1 The zebrafish retinotectal projection. (A) Diagram of the retinal axon pathway. Retinal axons navigate to the optic nerve head (1), pass through the optic nerve and exit the eye (2), cross the midline at the chiasm (3), and grow dorsally along the optic tract (4) to reach the tectum (5). (B) Dorsal view of a Tg(*isl2b:EGFP*)^{zc7} transgenic embryo, which specifically expresses EGFP in all RGCs, allowing a direct visualization of retinal projections. Courtesy of A. Pittman. A: anterior; P: posterior; OC, optic chiasm; OT, optic tectum. *Maximum intensity projection, confocal microscopy.* (A, B): dorsal views, anterior up.

navigate dorsally through the optic tract to reach their main target, the optic tectum (48 hpf), where they establish a topographic map, making connections according to their position in the retina (Fig. 2C–D). Axons originating from the more rostral retina project to the more posterior tectum, and axons from the dorsal retina project to the ventrolateral tectum. Interestingly, this ordering in the tectum can already be observed along the dorso-ventral axis in the optic tract: dorsal axons grow through the ventral branch of the tract, and ventral axons through its dorsal branch. Once in the tectum, retinal axons mature, arborize, and form synapses with their tectal targets.

Retinal axons encounter many guidance decision points along their pathway and respond to various attractive or repulsive cues to choose the right track. Many factors acting as road signs have been identified, but how retinal axons respond to them *in vivo* still remains poorly understood. Many laboratories, including ours, have developed tools for visualizing retinal axons during their navigation and modifying their nature or their environment to test specific functions. We describe here the different methods used for labeling and visualizing retinal axons, as well as several approaches for perturbing the retinotectal system. Many of these methods are also applicable to nonretinal axons. We finish with an overview of methods likely to be important in the future.

II. Visualizing Retinal Axons

Understanding how retinal projections develop requires specific labeling and precise visualization of retinal axons *in vivo*. Several methods can be used, depending on which part of the retinotectal pathway is studied, how many axons are observed, and whether the axons are observed live. Thanks to the optical transparency of zebrafish

embryos, transgenic lines expressing fluorescent proteins in RGCs can be used to visualize retinal axons as they develop. Lipophilic dyes are particularly useful to label specific groups of axons. Finally, transient expression of DNA constructs and *in vivo* electroporation are specially suited for labeling single axons and imaging them as they elongate. For all these approaches, precise imaging is best achieved using confocal microscopy.

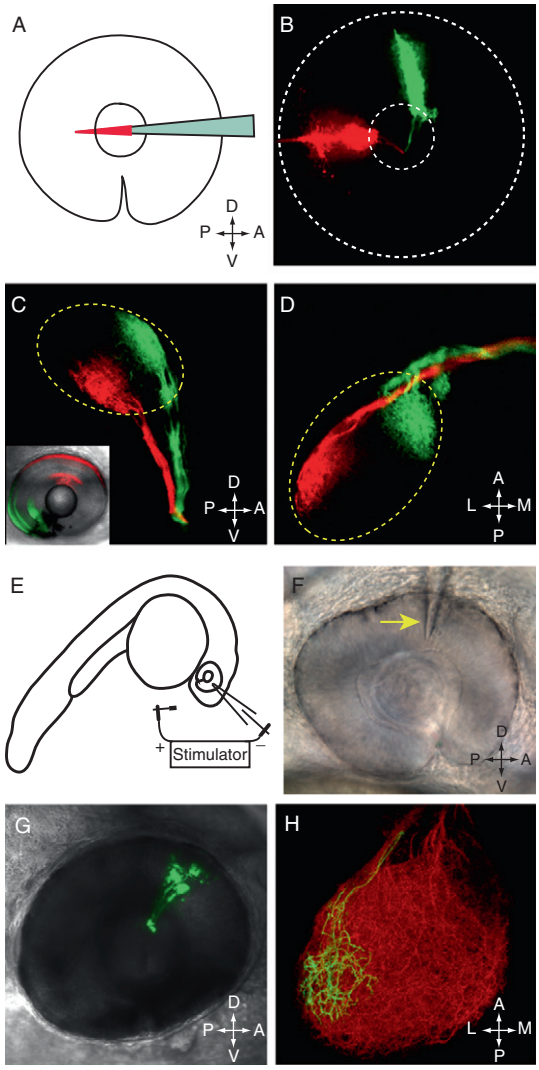

Fig. 2 *(Continued)*

A. Transgenic Lines

Several transgenic lines that express fluorescent proteins (FPs) under the control of RGC-specific promoters have been developed (Table I). Their main advantage is to allow clear and direct visualization of retinal projections in live embryos. Labeled embryos are simply obtained by crossing transgenic carriers. Depending on the promoter used, all RGCs or a subset of them are labeled. Promoters from the *isl2b* and *atoh7* (previously named *isl3* and *ath5*) genes drive transgene expression in all RGCs, allowing the visualization of all retinal axons (Fig. 1B, Masai *et al*., 2003; Pittman *et al*., 2008). In contrast, promoters from the *pou4f3* (previously named *brn3c*) gene can be used to label a subset of RGCs (Neumann and Nüsslein-Volhard, 2000; Xiao *et al*., 2005). For instance, the *pou4f3* promoter drives expression in RGCs that project mainly into one of the four retinorecipient layers of the tectum, allowing characterization of laminar targeting of retinal axons (Xiao *et al*., 2005).

Different FPs can be expressed to label RGCs. Enhanced green fluorescent protein (EGFP) is the most frequently used, as it is stable and particularly bright. RGCs can also be labeled in red using TagRFP or mCherry. Adding specific tags to the FP coding sequence allows labeling of specific cellular compartments such as the nucleus or the plasma membrane. For instance, the N-terminal palmitoylation sequence from GAP-43 (Moriyoshi *et al*., 1996) or the CAAX consensus motif from Ras (Choy *et al*., 1999) can target FPs to the plasma membrane, giving better labeling of axonal arbors.

Finally, other transgenic lines express the strong transcriptional activator Gal4-VP16, which drives the expression of DNA constructs containing a UAS (upstream activation sequence) control element (Köster and Fraser, 2001). These lines can be

Fig. 2 Methods for visualizing retinal axons. (A–D) Focal injection of dyes in the retina allows visualization of retinal axons exiting from the retina and making topographic connections in the tectum. (A) After removing lens, a dye-coated glass micropipette is briefly inserted in a peripheral direction into the RGC layer (method described in detail in Section II.G.1). (B) Lateral view of a 48 hpf eye focally injected with DiI (red) and DiO (green). Labeled retinal axons can be observed exiting from the retina. *Maximum intensity projection, confocal microscopy.* (C) Lateral view of a 4 dpf embryo topographically injected with DiI and DiO into the dorsonasal (DN) and ventrotemporal (VT) retina, respectively, using a vibrating-needle injection apparatus (Baier *et al*., 1996). Inset shows the sites of injection in the retina. DN (red) and VT (green) retinal axons navigate through the ventral and dorsal branches of the optic tract, respectively, and terminate topographically in the tectum. Yellow dashed line: tectal border. *Maximum intensity projection, confocal microscopy.* (D) Dorsal view of the projections showed in C. DN axons terminate in the posterolateral tectum, whereas VT axons innervate the antero-medial tectum. Yellow dashed line: tectal border. *Maximum intensity projection, confocal microscopy.* (E–H) *In vivo* single cell electroporation allows visualization of retinal arbors in the tectum. (E) Schematic representation of the electroporation setup: a 22–28 hpf embryo is mounted laterally under a compound microscope. A negatively charged glass microelectrode is filled with DNA solution and placed in the retina, with a positively charged ground electrode placed near the head. (F) DIC picture of the microelectrode (arrow) placed into the DN retina just prior to electroporation. *40× water immersion objective, compound microscope.* (G) Electroporated RGCs expressing GAP43-EGFP (green) in a live 5 dpf embryo mounted laterally with the lens removed. The EGFP image has been merged with a DIC image of the head. *Maximum intensity projection, confocal microscopy.* (H) Dorsal view of the contralateral tectum of the same embryo, with tectal neuropil visualized by *isl2b:mCherry-CAAX* transgene [red; Tg(*isl2b:mCherry-CAAX*)^{zc23}] and electroporated RGC axons and arbors visualized with GAP43-EGFP (green). *3D projection from Fluorender software, 40× water immersion objective, confocal microscopy.* A: anterior; P: posterior; D: dorsal; V: ventral; L: lateral; M: medial. (See Plate no. 1 in the Color Plate Section.)

Table I
Transgenic Lines that Label RGCs

Transgenic line	Previous names, ZFIN allele number	Retinal expression	Other expression	References
atoh7:GFP	*ath5:GFP, rw021*	Newborn RGCs	Forebrain, tectum	Masai *et al.* (2003), Poggi *et al.* (2005)
atoh7:mGFP	*ath5:mGFP, cu1*	"	"	Vitorino *et al.* (2009), Zolessi *et al.* (2006)
atoh7:mRFP	*ath5:mRFP, cu2*	"	"	Vitorino *et al.* (2009), Zolessi *et al.* (2006)
atoh7:Gal4-VP16	*zf138*	"	"	Maddison *et al.* (2009)
pou4f1-hsp70:GFP	*brn3a-hsp70:GFP, rw0110*	RGCs (likely a subset)	Tectum, habenula, cranial sensory ganglia	Aizawa *et al.* (2005), Sato *et al.* (2007)
pou4f3:mGFP	*brn3c:mGFP, s273t, s356t*	Subset of RGCs	Inner ear, lateral line neuromasts	Del Bene *et al.* (2008), Xiao *et al.* (2005)
pou4f3:Gal4VP16	*s311t*	"	"	Xiao and Baier (2007)
isl2b:GFP	*isl3:GFP, zc7*	All RGCs	Cranial ganglia Rohon-Beard neurons, a few cells in forebrain dorsal midbrain	Pittman *et al.* (2008)
isl2b:mGFP	*zc20*	"	"	Law and Chien (unpublished)
isl2b:mCherryCAAX	*zc23, zc25*	"	"	Pittman *et al.* (2008)
isl2b:Gal4VP16	*zc60*	"	"	Ben Fredj *et al.* (2010)
chrnb3b:GFP	*jt0021*	RGCs	Trigeminal ganglion, Rohon-Beard neurons, some tectal cells	Matsuda and Mishina (2004), Tokuoka *et al.* (2002), Yoshida and Mishina (2003)
-2.7shh:GFP	*t10*	RGCs	Amacrine cells, notochord, floor plate, pharyngeal arch endoderm, ventral forebrain	Neumann and Nüsslein-Volhard (2000), Nevin *et al.* (2008), Roeser and Baier (2003)

mGFP, Membrane-targeted GFP; RGCs, retinal ganglion cells.

particularly useful for expressing DNA constructs at high levels in a few RGCs (described in Section II.D).

B. Labeling with Antibodies

Alternately, antibodies can be used to label retinal axons. Although they cannot be employed for live visualization, they provide strong staining that can be useful to examine details or specific aspects of retinal axon navigation. Several antibodies have been widely used to label retinal axons using standard whole-mount antibody staining techniques. Anti-acetylated tubulin (Sigma, St. Louis, Missouri) recognizes a form of tubulin found in stable microtubules, and thus labels all axons. This staining has been used to visualize the earliest axons crossing the chiasm (Karlstrom *et al*., 1996) and to label axon bundles within the retina (Li *et al*., 2005). Zn-5 and zn-8 (Zebrafish International Resource Center, Developmental Studies Hybridoma Bank, Iowa City, Iowa) are two monoclonal antibodies, likely derived from the same hybridoma, that recognize the cell surface adhesion molecule Alcam-a (previously named neurolin/DM-GRASP, Laessing *et al*., 1994). Alcam-a is expressed by newly born RGCs that are added in successive peripheral rings around the retina, but turns off in central RGCs by 48 hpf (Laessing and Stuermer, 1996). Consequently, zn-5/8 staining is particularly appropriate to label retinal axons navigating within the retina to the optic nerve head. Finally, anti-GFP (Invitrogen, Carlsbad, California), anti-DsRed (which also recognizes mCherry, Clontech, Mountain View, California) and anti-TagRFP (Evrogen, Moscow, Russia) antibodies can be used to amplify the signal from FPs.

C. Labeling with Lipophilic Dyes

While transgenic lines and antibodies are appropriate for labeling a large population of axons, they cannot be used to visualize spatially specific sub populations of RGCs. Lipophilic carbocyanine dyes such as DiI, DiO, DiA, or DiD (Invitrogen) offer the great advantage of being easily injected in specific locations within the retina. Structurally, they consist of a fluorophore attached to two long aliphatic alkyl tails responsible for their insertion within membranes. Carbocyanine dyes are highly fluorescent in lipid bilayers, but weakly fluorescent in water. Once applied, they become incorporated into the plasma membrane and diffuse laterally, labeling the entire cell. These properties have made lipophilic dyes the tool of choice for anterograde and retrograde tracing of neurons in both live and fixed tissues (Honig and Hume, 1989).

DiI (red) and DiO (green) are the most commonly used. They can be applied using several methods. The first is to inject DiI or DiO dissolved in chloroform into the eye, which labels the entire projection ("whole eye fills"). This technique is particularly useful for studying guidance at the chiasm, as each eye can be labeled with a different color. It has been described previously (Hutson *et al*., 2004) and is not repeated here.

DiI and DiO can also be delivered into specific regions of the retina, so that only a subset of RGCs is labeled (Fig. 2A–D). In the second method, dyes dissolved in dimethylformamide are focally injected using a vibrating-needle injection apparatus (Baier *et al*., 1996; Trowe, 2000). DiI or DiO is loaded in a reservoir through which

passes a tungsten needle. Fixed larvae are mounted in an agarose form. A small loudspeaker vibrates the needle, transporting dye to its tip, where the dye precipitates in the embedded tissue. This method has the advantage of labeling many embryos reproducibly and has been used to analyze projection topography in the tectum and axon ordering in the tract (Karlstrom *et al*., 1996; Lee *et al*., 2004; Trowe *et al*., 1996). However, the custom-built apparatus is not widely available. The third technique uses a dye-coated microneedle to focally deposit dye into the retina. The needle is coated with dye and can be reused several times. It does not require any specialized apparatus and can be used to label very few cells. We describe this method in Section II.G.1. A final method is to focally inject DiI along the retinal pathway to retrogradely label RGCs. Although it is difficult to inject dye precisely enough, this technique can be used to visualize RGC morphology and organization within the retina (Mangrum *et al*., 2002).

D. Transiently Expressing DNA Constructs

Whereas lipophilic dyes can easily label a subset of RGCs, they are more difficult to use for single axons. These can be better visualized by transiently expressing DNA constructs encoding FPs. Plasmids injected at the one cell stage are expressed mosaically, labeling a few cells randomly. Expression can be targeted to RGCs using the *atoh7* or *isl2b* promoters (Masai *et al*., 2003; Pittman *et al*., 2008). This method has been used to visualize single retinal arbors (Campbell *et al*., 2007) and RGC dendritic outgrowth (Mumm *et al*., 2006). Alternatively, constructs containing a UAS element upstream of an FP coding sequence can be injected into transgenic embryos expressing Gal4-VP16 in RGCs (Table I). The Gal4/UAS system amplifies FP expression and gives better labeling. While DNA methods are very useful for labeling single axons, they cannot yet be used to target specific RGC subtypes or RGCs in particular locations, since the required enhancers have not yet been identified.

E. *In Vivo* Single Cell Electroporation

Another way to label individual axons is *in vivo* single cell electroporation (Fig. 2E–H). Although technically demanding, this powerful approach offers the possibility of delivering DNA constructs or dextran-coupled indicators to individual RGCs or RGCs in specific locations in the retina. We have used it to visualize projections and arborizations of individual dorsonasal RGCs (Pittman *et al*., 2010). In this approach, an applied voltage generates an electric field across cells in the retina, breaking down the plasma membrane and creating transient pores through which negatively charged DNA molecules move into the cell. Briefly, embryos are mounted laterally on a glass slide in agarose that is windowed to expose the eyes, covered with medium, and viewed under a 40× water immersion objective. A glass microelectrode filled with DNA or tracer solution is poked into the retina with a micromanipulator, and a voltage train applied. Embryos are then unmounted and raised. This approach allows coelectroporation of several indicators or constructs into the same cell, allowing both visualization and perturbation experiments. We describe this technique in detail in Section II.G.2.

F. Time-Lapse Imaging

Time-lapse imaging of RGC axons is crucial to understand their response to the environment. It has been used by many investigators to monitor axons' behavior (Campbell *et al*., 2007; Hutson and Chien, 2002; Kaethner and Stuermer, 1992; O'Brien *et al*., 2009; Schmidt *et al*., 2000) and can be used with all the labeling techniques described above, except for antibody labeling. Confocal or two-photon microscopy is most appropriate for time-lapse imaging and can be performed with an upright or inverted microscope. Several protocols have been previously described, so we do not discuss them here (Campbell *et al*., 2007; Hutson and Chien, 2002; Hutson *et al*., 2004; Meyer and Smith, 2006).

G. Protocols for Labeling Methods

Here we describe detailed protocols for focal injections of lipophilic dyes in the retina and for *in vivo* single cell electroporation.

1. Method 1: Precise Labeling with Intraretinal Injection of Lipophilic Dyes

This method uses glass microneedles coated with lipophilic carbocyanine dyes to focally deposit dye into the retina (Fig. 2A–B). It can be used to target specific locations in the retina and label very few cells. It was originally developed by Torsten Trowe (2000).

a. Solutions Needed

- DiI or DiO crystals (Molecular Probes)
- 4% PFA (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4)
- 1% low-melt agarose in PBS (phosphate-buffered saline)
- 50% and 80% glycerol in water

b. Protocol

1. Fix zebrafish embryos at required stage in 4% PFA at 4°C for at least 12 h. For growth cone labeling, fix at room temperature for the first 2 h.
2. Use glass capillary with an outer diameter of 1.0 mm and an inner diameter of 0.58 mm to prepare the micropipette for injections. Pull the capillary to a final taper length of 9.0 mm and a tip size of 2 μm. To coat the micropipette with dye, place a few dye crystals on a cover glass and melt them at 100°C on a hot plate. Dip the tip of the micropipette horizontally into the dye paste and roll it to cover the tip equally on all sides. Wipe off as much dye from the tip as possible onto the cover glass.
3. Prepare 30 ml of 1% low-melt agarose and keep on heating block at 45°C to prevent from solidifying. Use a Petri dish lid to embed embryos for dye injection. Coat bottom with a thin layer of 1% low-melt agarose and let solidify. Transfer embryos with as little PFA as possible onto the agarose. Cover embryos with a drop of 1% low-melt agarose and orient them in a lateral position.
4. When the agarose covering is solid, use a sharpened tungsten needle to remove the top-facing lens by carefully cutting the skin covering the eye in a circle along the

border between lens and retina. The lens will become loose and can now be easily removed. The resulting hole should be refilled with 1% low-melt agarose. The embryos are now ready to be injected with the dye.

5. Use a standard pipette holder and three-axis micromanipulator to hold the dye-coated micropipette. Insert it into the RGC layer by placing it in the empty lens cup and advancing in a peripheral direction at a roughly 45° angle (Fig. 2A). Leave the needle in the eye for not more than 2 s to ensure a small injection site and labeling of only a few axons. The coated micropipette can be reused for several injections before it has to be coated again with fresh dye.
6. After finishing the injections, cover embedded embryos with 1× PBS or water to avoid drying. This step also washes off excessive dye. Store the embryos for a few hours at room temperature for fast diffusion of the dye, or keep them at 4°C overnight if slower diffusion is desired. Long incubation times can result in nonspecific diffusion of the dye within the eye, which can prevent clear imaging results later on.
7. Recover embryos from the agarose bed using forceps. Place them in a microfuge tube and wash them in 1× PBS. Transfer embryos to 50% glycerol/H_2O and incubate them for 3 h at 4°C with agitation. Change the medium to 80% glycerol/H_2O, and store embryos at 4°C overnight. Now that they are cleared, embryos can be mounted for confocal imaging in 80% glycerol between two coverslips (Fig. 2B).

2. Method 2: Single Cell *In Vivo* Electroporation

In vivo focal electroporation is used to deliver tracers or transgenes into single RGCs. It can target several or individual cells in precise topographic positions within the retina (Fig. 2E–H). An electric field applied across an RGC progenitor creates transient pores in the plasma membrane through which negatively charged DNA molecules move into the cell. We have used the protocol detailed here to image single RGC arbors in the tectum (Pittman *et al.*, 2010); it was slightly modified from a previous method for imaging habenular neurons (Bianco *et al.*, 2008).

a. Solutions Needed

- E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 1.7 mM $NaHCO_3$)
- 0.1 mM phenylthiourea (PTU) in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$)
- tricaine stock (0.4% tricaine, 10 mM HEPES, pH 7.4)
- 1% low-melt agarose in E2/GN/tricaine (10 µg/ml gentamicin in E2 medium, 0.02% tricaine)

b. Protocol

1. Raise embryos at 28.5°C in E3 medium containing 0.1 mM PTU to inhibit pigment formation, and dechorionate them between 22 and 28 hpf. Anesthetize embryos by adding tricaine to a final concentration of 0.02%. Mount laterally in a drop of 1% low-melt agarose in E2/gentamycin/ tricaine, in wells built with quick-hardening

epoxy on a glass microscope slide. Expose the eye by cutting a small window in the agarose with forceps, and cover the embryo with E3-PTU + 0.02% tricaine.

2. After mounting the embryo, place the glass slide under a 40× water immersion objective on an upright compound microscope. Place an Ag/AgCl cathode in the overlying buffer near the head of the embryo. Backfill a glass microelectrode (1–3 µm diameter tip) with 2 µl of solution containing the tracer or DNA (final concentration of 1–3 µg/µl in water or 10 mM Tris-HCl, pH 8.5), and place it in the retina using a micromanipulator. Use a stimulator to deliver 1 s trains of 2 ms negative-going square pulses at 200 Hz, 30–50 V (reverse polarity and use 3–5V for positively charged tracers). An effective train will cause a visible rippling effect (tissue response) in the tissue surrounding the microelectrode tip when the voltage train is applied. A clogged needle will result in a less pronounced tissue response. A "pop" will occasionally appear in the tissue in response to a voltage train, resulting in an ineffective electroporation. While the exact cause of the pop is not known, it occurs less often with a lower DNA concentration and a lower voltage. Each cell is targeted with 3–5 trains, and several cells can be targeted per eye. After electroporation, the embryo is removed from the agarose and raised in E3 + PTU at 28.5°C
3. FP expression can be seen in electroporated RGCs in the eye under a fluorescent dissecting microscope by 12 h after electroporation. Corresponding axons can be visualized in the contralateral optic tract and tectum under a 40× water objective on a compound microscope, or by confocal microscopy. Labeled axons are best observed from a dorsal view in the contralateral tectum, or from a lateral view in the contralateral optic tract after removal of the contralateral eye. Time-lapse imaging can also be performed.

III. Perturbing the Retinotectal System

Experimental manipulations perturbing axons or their environment are crucial to understand how and by which molecular mechanisms retinotectal projections develop. Many important factors have been discovered through the generation and characterization of mutants with retinotectal defects isolated in large-scale genetic screens. In addition, several approaches including DNA or antisense morpholino oligonucleotide (MO or "morpholino") injections, heat shock experiments, or transplants can be used to assess the function of a particular protein.

A. Retinotectal Mutants

The first mutants with retinotectal defects were obtained from a large genetic screen performed in Tübingen in the 1990s (Karlstrom *et al*., 1996; Trowe *et al*., 1996). Topographic injections of DiI and DiO in the retina were used as an assay to identify mutants with defects in retinal axon pathfinding, sorting in the tract, and topography in the tectum. Almost all the genes affected in these mutants have now been identified, allowing the discovery of crucial regulators of axon guidance or brain patterning, including the receptors *robo2* and *patched1*, the transcription factor *lhx2*, and the

Table II
Retinotectal Pathfinding Mutants

Mutant name (abbreviation)	Region in which pathfinding affected	Gene	Brain defect?	References
acerebellar (ace)	Chiasm, anterior projection, optic tract, topography	*fgf8*	Yes	Picker *et al.* (1999), Shanmugalingam *et al.* (2000)
astray (ast)	Chiasm, anterior projection, optic tract, tectum aroborization	*robo2*	No	Campbell *et al.* (2007), Fricke *et al.* (2001), Hutson and Chien (2002), Karlstrom *et al.* (1996)
bashful (bal)	Retinal exit, anterior projection	*laminin α1*	Yes	Karlstrom *et al.* (1996), Paulus and Halloran (2006)
belladonna (bel)	Midline crossing	*lhx2*	Yes	Karlstrom *et al.* (1996), Seth *et al.* (2006)
beyond borders (beyo)	Confinement to tectal neuropil	*?*	Yes	Xiao *et al.* (2005)
blind date (blin)	Tectum innervation	*?*	No	Muto *et al.* (2005), Xiao *et al.* (2005)
blowout (blw)	Midline crossing eye shape	*patched 1 (ptc)*	Yes	Karlstrom *et al.* (1996), Lee *et al.* (2008)
blue kite (bluk)	Tectum innervation	*?*	No	Xiao *et al.* (2005)
blumenkohl (blu)	Expanded terminations	*slc17a6b (glutamate transporter)*	No	Smear *et al.* (2007), Trowe *et al.* (1996)
bogus journey (boj)	Midline crossing	*?*	?	Muto *et al.* (2005)
boxer (box)	Tract sorting, crossing in posterior commissure	*extl3*	No	Karlstrom *et al.* (1996), Lee *et al.* (2004), Trowe *et al.* (1996)
breaking up (brek)	Confinement to tectal neuropil	*?*	No	Xiao *et al.* (2005)
chameleon (con)	Retinal exit, midline crossing	*dispatched homolog 1 (dips1)*	Yes	Karlstrom *et al.* (1996), Nakano *et al.* (2004)
clueless (clew)	Tectum innervation	*?*	No	Xiao *et al.* (2005)
coming apart (coma)	Optic tract, tectum innervation	*?*	No	Xiao *et al.* (2005)
cyclops (cyc)	Midline crossing	*nodal related-2 (ndr2)*	Yes	Karlstrom *et al.* (1996), Rebagliati *et al.* (1998), Sampath *et al.* (1998)
dackel (dak)	Tract sorting crossing in posterior commissure	*ext2*	No	Karlstrom *et al.* (1996), Lee *et al.* (2004), Trowe *et al.* (1996)
dark half (darl)	Ventral branch of the optic tract missing, topography	*gdf6a*	No	Gosse and Baier (2009), Muto *et al.* (2005)
detour (dtr)	Midline crossing	*gli1*	Yes	Karlstrom *et al.* (1996, 2003)
dragnet (drg)	Laminar specificity in the tectum	*collagen IVa5 (col4a5)*	No	Xiao and Baier (2007), Xiao *et al.* (2005)
esrom (esr)	Midline crossing, termination	*MYC binding protein 2 (mycbp2) or PAM*	No	D'Souza *et al.* (2005), Karlstrom *et al.* (1996), Trowe *et al.* (1996)
excellent adventure (exa)	Targeting defect in the tectum	*?*	?	Muto *et al.* (2005)

fuzz wuzzy (fuzz)	Confinement to tectal neuropil	*?*	No	Xiao *et al.* (2005)
gnarled (gna)	Tectal entry, tectal misrouting	*?*	Yes	Trowe *et al.* (1996), Wagle *et al.* (2004)
grumpy (gup)	Anterior projection, midline crossing	*laminin β1*	Yes	Karlstrom *et al.* (1996), Parsons *et al.* (2002)
iguana (igu)	Midline crossing	*DAZ interacting protein 1 (dzip1)*	Yes	Karlstrom *et al.* (1996), Sekimizu *et al.* (2004), Wolff *et al.* (2004)
late bloomer (late)	Delayed innervation of the tectum	*?*	No	Xiao *et al.* (2005)
no isthmus (noi)	Chiasm, anterior projection, tectal bypass	*pax2a*	Yes	Brand *et al.* (1996), MacDonald *et al.* (1997), Trowe *et al.* (1996)
macho (mao)	Expanded terminations	*?*	No	Gnuegge *et al.* (2001), Trowe *et al.* (1996)
michikusa (mich)	Ectopic arbor after crossing the midline	*?*	?	Muto *et al.* (2005)
missing link (miss)	Pretectal targets (AF4, AF9) absent or reduced	*?*	?	Muto *et al.* (2005)
nevermind (nev)	Tract sorting, D-V topography	*cyfip2*	No	Pittman *et al.* (2010), Trowe *et al.* (1996)
odysseus (ody)	Intraretinal guidance defects	*cxcr4b*	No	Knaut *et al.* (2003), Li *et al.* (2005)
parachute (pac)	Ipsilateral projection\; entering chiasm area	*N-cadherin*	Yes	Lele *et al.* (2002), Masai *et al.* (2003)
pinscher (pic)	Tract sorting, crossing in posterior commissure	*papst1 (sulfate transporter)*	No	Clément *et al.* (2008), Karlstrom *et al.* (1996), Trowe *et al.* (1996)
shirli-myrli (shir)	Delayed innervation of the tectum	*?*	No	Muto *et al.* (2005)
sleepy (sly)	Anterior projection; midline crossing	*laminin γ1*	Yes	Karlstrom *et al.* (1996), Parsons *et al.* (2002)
smooth muscle omitted (smu)	Midline crossing	*smoothened (smo)*	Yes	Chen *et al.* (2001), Varga *et al.* (2001)
sonic-you (syu)	Retinal exit, midline crossing	*sonic hedgehog (shh)*	Yes	Brand *et al.* (1996), Schauerte *et al.* (1998)
space cadet (spc)	Retinal exit, midline crossing	*?*	No	Karlstrom *et al.* (1996), Lorent *et al.* (2001)
tarde demais (tard)	Delayed innervation of the tectum	*?*	No	Xiao *et al.* (2005)
umleitung (uml)	Midline crossing	*?*	Yes	Karlstrom *et al.* (1996)
vertigo (vrt)	Delayed innervation of the tectum	*?*	No	Xiao *et al.* (2005)
walkabout (walk)	Pretectal target AF4 overinnervated	*?*	?	Muto *et al.* (2005)
who cares (woe)	Tract sorting, D-V topography	*?*	No	Trowe *et al.* (1996)
you-too (yot)	Midline crossing	*gli2*	Yes	Karlstrom *et al.* (1996, 1999)

?=not known

adhesion molecule *N-cadherin* (see Table II for a complete listing of these mutants). While some genes such as *astray (robo2)* primarily affect axon navigation, others such as *ace (fgf8)* disrupt brain patterning, resulting in mispresented axon guidance cues. More recently, a new screen has been performed using the *pou4f3:mGFP* transgenic line expressing membrane-targeted GFP (mGFP) in a subset of RGCs (Xiao *et al*., 2005). This approach allowed the identification of novel mutants with various defects in tectum innervation (Table II). Two mutants from this screen have been cloned, revealing new functions for *gdf6a* and *collagenIVa5* in regulating eye dorso-ventral patterning and tectum laminar targeting, respectively (Gosse and Baier, 2009; Xiao and Baier, 2007). Finally, a recent screen using behavioral assays identified mutants with disrupted response to visual motion and/or impaired background adaptation (Muto *et al*., 2005). Some of these mutants also have abnormal retinotectal projections or a lack of RGCs that are likely responsible for their phenotype. Identifying the mutations generated in these newer screens will give new clues about the factors involved in retinal axon guidance.

B. Injecting DNA or Morpholinos

A common approach to characterize protein function in zebrafish is to inject stable MOs into one-cell stage embryos. MOs inhibit either protein translation when targeted near the start codon of mRNAs (Nasevicius and Ekker, 2000) or splicing of the pre-mRNAs when they are targeted to exon–intron or intron–exon boundaries (Draper *et al*., 2001). Under good conditions, MOs can quickly reveal required functions for a targeted gene, though their use is subject to several caveats, including loss of efficacy as they are diluted during development (Eisen and Smith, 2008). We took advantage of this dilution with an MO against the transcription factor *atoh7* to specifically block differentiation of early- but not late-born RGCs, allowing the functional analysis of isotypic interactions between pioneer and follower axons during navigation (Pittman *et al*., 2008).

Alternatively, DNA constructs encoding dominant negative forms of the protein of interest can be transiently or stably expressed. Temporal or spatial control can be provided by the *hsp70l* heat shock promoter (see following section) or cell-specific promoters, respectively. Similarly, gain-of-function experiments can be performed by misexpressing genes of interest at specific times or locations. For greater precision, DNA constructs or MOs can be delivered to individual RGCs by *in vivo* cell electroporation (described in Section II.E), allowing functional studies at single-cell resolution (Pittman *et al*., 2010).

C. Using Heat Shock to Induce Misexpression

A powerful technique to misexpress genes in a temporally or spatially controlled manner is to use heat shock. This approach is particularly useful for studying genes with both early and late roles during development. Heat shocks can be performed after transient injection of DNA constructs or on stable transgenic lines. The *hsp70l* promoter

is an inducible element that drives strong gene expression in response to a temperature shift from 28.5°C (normal rearing temperature) to 37–40°C (Halloran *et al*., 2000). Global heat shocks have been widely used to induce ubiquitous gene expression in embryos at specific times. The exact heat shock duration and temperature depend on the age of the embryo, the transgene to be expressed, and the level of expression desired. For instance, raising the temperature to 42°C for 5 min can induce detectable transgene expression in 20 hpf embryos (Thummel *et al*., 2005).

Recently, we developed a technique using a sharpened soldering iron to induce focal heat shocks in restricted regions of the embryo (Hardy *et al*., 2007). For this approach, a copper soldering iron tip with a diameter of 15 µm is heated to 60°C and put directly in contact with the embryo for 3 min. A perfusion chamber keeps fluid flowing over the embryo during heat shock, thereby preventing heating of the medium and restricting the area of activation. This method is rapid and easy, allows the targeting of ~100 µm patches of tissue, and can be used in a variety of tissues and stages. A detailed protocol has been described (Hardy *et al*., 2007). Even more recently, Rolf Karlstrom's group developed another focal heat shock method using an optical fiber to deliver energy to a localized region (Placinta *et al*., 2009).

D. Transplanting to Test Cell Autonomy of Gene Function

Transplanting cells or tissues is a powerful approach to test cell autonomy of gene function. Different types of transplant can be performed depending on the question (e.g., transplanting all RGCs, or RGCs in specific parts of the retina; labeling donors, hosts, or both labeled). A tricky but elegant approach is to transplant entire eye primordia, yielding mosaic embryos in which the whole eye comes from the donor while the rest of the embryo is derived from the host. A main advantage of this approach is that all retinal axons coming from the transplanted eye share the same genotype and are not influenced by interactions with host retinal axons, as these have been removed. We used eye transplants to demonstrate that *robo2* acts eye-autonomously to regulate retinal axon guidance (Fricke *et al*., 2001). A detailed protocol has been previously described (Hutson *et al*., 2004).

Alternatively, early transplants at blastula stage can be used to test cell autonomy (Ho and Kane, 1990). These are easy to perform and allow quite effective targeting of the retina (Moens and Fritz, 1999). Cells are removed from donor embryos between 4 and 6 hpf and replaced into the animal pole of host embryos. The resulting mosaic embryos display clones of RGCs in the retina, as well as some clones of cells in the brain. An abbreviated protocol is given below. While the presence of donor cells in the brain may make results harder to interpret, this approach is the easiest way to generate mosaic embryos with RGCs from different genetic backgrounds. However, it cannot be employed to target RGCs from or to specific regions within the retina.

Instead, transplants at a later stage are required. We have recently begun to use a technique for transplanting RGCs in a topographic manner (Fig. 3; inspired by Masai *et al*., 2003). Donor and host embryos labeled with different transgenes are grown to 30–33 hpf, when the first RGCs are specified. After mounting embryos laterally in

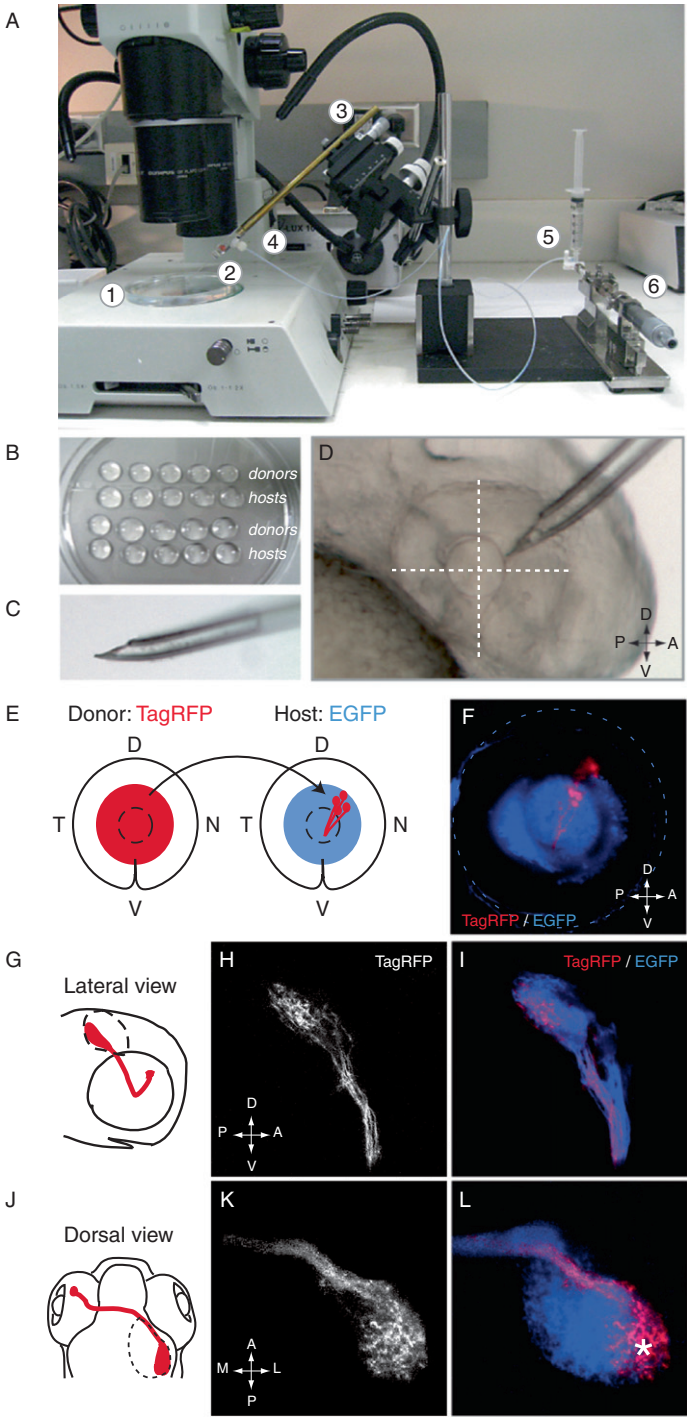

Fig. 3 *(Continued)*

agarose, donor RGCs are precisely removed from a specific location in the retina with a 40 µm glass micropipette and replaced at the same position in the host retina. The transplant is considered successful if, after raising the host, the transplanted RGCs are observed in the correct area of the retina (from a lateral view), and if in control conditions their arbors terminate in the appropriate part of the tectum (from a dorsal view). We obtain ~25% successful transplants with this approach and provide a detailed protocol below.

E. Protocols for Transplants

1. Method 3: Blastula Transplants

Since blastula stage transplants have been explained in detail elsewhere (Ho and Kane, 1990; Kemp *et al*., 2009), only a succinct description of the method is provided here.

1. Donor embryos are injected at the one-cell stage with 5% Alexa-488 dextran or rhodamine dextran (10,000 MW) as a lineage marker. The light color from the dextran helps to distinguish donors from hosts during later steps. We use agarose-groove dishes for the injections (mold TU-1, Adaptive Science Tools, Worcester, Massachusetts; 1% agarose w/v in E2 or E3 embryo medium). Donor and host embryos are raised at 28.5°C until the sphere (4 hpf) or shield stage (6 hpf).
2. While waiting for the embryos to develop, pull and bevel standard wall, non-filament capillaries for use as transplant needles and prepare an agarose transplant dish (single-well mold; mold PT-1, Adaptive Science Tools; Kane and Kishimoto, 2002).
3. Dechorionate donor and host embryos. Use a clean fire-polished large-bore Pasteur pipette to transfer one donor and four hosts into each row of the transplant dish using an air-filled syringe and fire-polished transplantation pipette. Remove cells

Fig. 3 Perturbing the retinotectal system with late topographic transplants. (A) Embryos are mounted laterally in drops of low-melt agarose deposited on a dish lid that is then placed under a dissecting microscope (1). The transplant needle is mounted in a micropipette holder (2), itself mounted onto a three-axis micromanipulator (3) placed next to the microscope. The micropipette holder is connected via a tube filled with mineral oil (4) to an oil-filled Hamilton syringe with a micrometer drive (6). The syringe is attached by a three-way stopcock to a reservoir filled with mineral oil (5). (B) Donor and host embryos mounted laterally in low-melt agarose drops. Embryos are arranged so that each donor is close to its respective host. (C) The transplant needle has a 40 µm diameter opening with a sharp tip that is slightly bent (around 20°). (D) The transplant needle is inserted into the dorsonasal retina, close to the lens, at a 45° angle. The bend of the needle tip is facing upward, so that ventral RGCs cannot be drawn up. (E) Dorsonasal (DN) RGCs from an *isl2b:TagRFP* donor are isotopically transplanted into the DN retina of an *isl2b:EGFP* host between 30 and 33 hpf. Their axonal projections are then visualized at 4 dpf by live confocal microscopy. (F) Lateral view of a WT *isl2b:EGFP* host eye in which WT TagRFP-positive RGCs have been transplanted. GFP is shown as blue for the best visualization. (G, J) Projections of DN donor axons observed in transplants in lateral (G) and dorsal (J) views. (H, I) Lateral view of TagRFP-positive projections at 4 dpf. DN donor axons navigate along the ventral branch of the tract to reach the tectum. (K, L) Dorsal view of the same projections. DN donor axons project to the posterolateral part of the host tectum (asterisk). *F, H, I, K, L: confocal maximum intensity projections*. (See Plate no. 2 in the Color Plate Section.)

from each donor embryo and transplant 20–50 cells into the animal pole of each corresponding host. While the origin of the transplanted cells is not important, the location where they are placed into the host is crucial. A fate map of the 6 hpf embryo can be used as a reference (Woo *et al.*, 1995).

4. After transplantation, transfer the agarose dish carefully to the 28.5°C incubator. During gastrulation, donor cells will spread out and form a mosaic patch of fluorescently labeled cells; choose those in which this patch includes cells in the eye. Once embryos have developed to bud stage, it is safe to remove them from the transplant dish and put them in 4-well or 24-well dishes. For experiments in which mutant cells are transplanted, donors should be kept together with their respective hosts until genotyped, either by PCR or by mutant phenotype. If necessary, hosts can be genotyped as well.

2. Method 4: Late Topographic Transplants

While blastula transplants are useful for testing functional cell autonomy and can be easily performed, they cannot target RGCs within specific regions of the retina. Testing the roles of genes specifically expressed in the dorsal or ventral retina, for instance, requires transplanting at later stages in a topographic manner. Here, we describe a detailed protocol for transplanting dorsonasal RGCs into the host dorsonasal retina. These transplants are performed between 30 and 33 hpf, when the first RGCs are specified and have acquired their positional identity within the retina. Donor and host embryos are labeled with *isl2b:TagRFP* and *isl2b:EGFP* transgenes, respectively, so that axons of transplanted RGCs and their projections can be easily visualized by live confocal miscroscopy at 4 days post-fertilization (dpf).

a. Solutions Needed

- E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM KH_2PO_4, 1.7 mM $NaHCO_3$)
- 0.1 mM phenylthiourea (PTU) in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$)
- tricaine stock (0.4% tricaine, 10 mM HEPES, pH 7.4)
- 1% low-melt agarose in E2/GN/tricaine (10 µg/ml gentamicin in E2 medium, 0.02% tricaine)

b. Protocol

1. The transplant needle is prepared in advance and can be reused several times. The quality of its preparation is the most important parameter for successful transplants. Pull standard wall, non-filament capillaries and polish them using a microforge, so that the tip displays a 20° angle with a 40 µm diameter opening (Fig. 3C).
2. Raise embryos at 28.5°C in E3 medium containing 0.1 mM PTU to inhibit pigment formation, and dechorionate them between 22 and 28 hpf. At 30 hpf, anesthetize embryos by adding tricaine to a final concentration of 0.02%. Mount laterally in a

drop of 1% low-melt agarose in E2/gentamycin/tricaine deposited on the lid of a Petri dish (Fig. 3B). Donors and hosts should be arranged in lines, so that each donor is close to its respective host. Once the drops have solidified, fill the Petri dish with PTU-E3/tricaine and position it under a dissecting scope.

3. Prepare the transplant setup (Fig. 3A): an oil-filled Hamilton syringe with a micrometer drive is connected by a three-way stopcock to a reservoir filled with mineral oil and to a micropipette holder through flexible plastic tubing. It is important to fill the system completely with mineral oil and ensure that air bubbles have been eliminated (air bubbles impair the ability to control suction and pressure). The transplant pipette is mounted in the micropipette holder, itself mounted onto a three-axis micromanipulator positioned next to the dissecting scope.
4. Using the micromanipulator, bring the transplant pipette near the dorsonasal retina, with a 45° angle (Fig. 3C). Make sure that the needle opening is facing upward, so that ventral RGCs cannot be drawn up into the needle. Insert the needle into the dorsonasal retina close to the lens, and slowly and carefully suck up 40–100 cells into the needle. At this stage, the fluorescence of the transgene expressed in RGCs is not yet visible, so the fraction of RGCs among the removed cells can vary. After cells have been taken up, reverse the pressure in the needle to stop the suction, and remove the needle from the donor eye. Insert the needle into the host retina in a similar way, and slowly expel the cells with as little medium as possible. After transplantation, let embryos recover for few minutes, remove them from the agarose, and raise them in E3 + PTU at 28.5°C in 24-well plates. Axons of transplanted RGCs can then be observed after 48 hpf by live imaging.

IV. Future Directions

The approaches developed over the past decade have greatly improved our ability to label and visualize the retinotectal projection *in vivo,* as well as to perform functional assays for understanding the molecular mechanisms that control its development. Nevertheless, novel techniques will be required for observing retinal axons in greater detail and to ask new biological questions.

Three methods already used in other systems are currently being adapted to study new aspects of zebrafish retinotectal system development. The Brainbow approach, initially developed in mice, allows labeling and mapping of neurons with a wide range of colors by randomly varying the levels of red, green, and blue FPs expressed in individual neurons (Livet *et al*., 2007). It has been used to reconstruct the architecture of neuronal circuits in different systems and will be a powerful tool for analyzing sorting of retinal axons in the tract as well as topographic mapping in the tectum. A second approach is to use enhancer trap (ET) screens to isolate lines expressing transgenes in specific subsets of neurons. For instance, new lines with interesting expression patterns in the tectum have recently been produced with a Gal4 ET screen (Scott and Baier, 2009). Such an approach will potentially allow the identification of new lines driving expression in specific regions of the retina (Picker *et al*., 2009) or RGC subtypes. Finally, calcium

imaging has been used *in vitro* to measure growth cone responses to guidance cues (Guan *et al*., 2007; Tojima *et al*., 2010). Adapted to zebrafish, it will give the ability to monitor, *in vivo*, the activity of retinal axons as they elongate. Combined together, these emerging techniques will improve our ability to examine retinal axons as they navigate, shedding new light on axon guidance *in vivo*.

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CHAPTER 3

SONIC HEDGEHOG CONTROLS INTRARETINAL AXON PATHFINDING THROUGH REGULATION OF CXCL12A IN THE OPTIC STALK

The following chapter is written in the form of a peer-reviewed manuscript coauthored by Cornelia Stacher Hörndli and Chi-Bin Chien, submitted to *Development*

Summary

Successful axon pathfinding requires both correct patterning of tissues, which will later harbor axonal tracts, and precise localization of axon guidance cues along these tracts at the time of axon outgrowth. Retinal ganglion cell (RGC) axons grow towards the optic disc in the central retina, where they turn to exit the eye through the optic nerve. Normal patterning of the optic disc and stalk and the expression of guidance cues at this choice point are necessary for the exit of RGC axons out of the eye. Sonic hedgehog (Shh) has been implicated in both patterning of ocular tissue and direct guidance of RGC axons. Here, we examine the precise spatial and temporal requirement for Hedgehog (Hh) signaling for intraretinal axon pathfinding and show that Shh acts to pattern the optic stalk in zebrafish but does not guide RGC axons inside the eye directly. We further reveal an interaction between the Hh and chemokine pathways for axon guidance and show that *cxcl12a* functions downstream of Shh and depends on the latter for its expression at the optic disc. Together, our results support a model in which Shh acts in RGC axon pathfinding indirectly by regulating axon guidance cues at the optic disc through patterning of the optic stalk.

Introduction

In the journey of an RGC axon, the first important steps for correct pathfinding to the optic tectum are its extension towards the optic disc and, after reaching this choice point, turning into the optic stalk to exit the eye through the optic nerve. When RGC axons fail to turn at the optic disc, they project within the eye and become trapped. Several molecules have been implicated in guidance of RGC axons out of the eye, acting either

through patterning of ocular tissue, direct axon guidance, or modulation of additional guidance cues (Deiner et al., 1997; Schauerte et al., 1998; Dakubo et al., 2003; Li et al., 2005). Shh is one factor that regulates ocular patterning in multiple species. A gradient of Shh along the proximodistal axis, formed by *shh* expression in the floorplate and notochord, has been implicated in the specification of early eye tissue into optic stalk and retina through the regulation of Pax2 and Pax6 expression domains (Ekker, et al. 1995; Macdonald et al., 1995; Perron et al., 2003). Additionally, Shh expressed by RGCs is required for normal formation of astrocytes at the optic disc and stalk in mouse, with subsequent effects on RGC axon guidance (Wallace and Raff, 1999; Dakubo et al., 2003, 2008). However, there is also evidence for direct guidance of axons by Shh. Both *in vitro* and *in vivo* studies in mouse and chick have implicated Shh in direct RGC axon guidance by signaling through non-canonical Hh pathways independent of target gene transcription (Trousse et al., 2001; Kolpak et al., 2005, 2009; Sánchez-Camacho and Bovolenta, 2008; Fabre et al., 2010; Gordon et al., 2010).

In this study, we used zebrafish as a model to determine if Shh regulates intraretinal axon pathfinding indirectly through tissue patterning or directly as a guidance molecule. We used embryological, pharmacological, and genetic approaches to examine the spatial and temporal requirement for Shh in retinal axon pathfinding. Our results strongly suggest an indirect requirement for Hh signaling in intraretinal pathfinding through patterning of the optic stalk and argue against a direct requirement for Shh in intraretinal axon guidance in zebrafish. We further show that Shh regulates the expression of several genes at the optic stalk and disc. One of these, *cxcl12a*, has previously been implicated in intraretinal axon guidance in zebrafish (Li et al., 2005). Using genetic analysis, we show

an interaction between the Hh and chemokine signaling pathways for RGC axon guidance out of the eye. Altogether, our study leads us to propose that Hh signaling during early optic vesicle development is required for proper patterning of the optic stalk and the correct expression of downstream guidance molecules, specifically *cxcl12a*, at the optic disc, which direct RGC axons out of the eye.

Materials and methods

Mutant and transgenic lines

All fish were of Tü or TL strains. Embryos were raised at 28.5°C in 0.1 mM phenylthiourea (PTU, to inhibit pigment formation). The mutant alleles used are *shha*^{tbx392} (Schauerte et al., 1998), *smo*^{hi1640Tg} (Chen et al., 2001) and *cxcl12a*^{ny054} (Valentin et al., 2007). Transgenic lines used were *Tg(-17.6isl2b:GFP)*^{zc7} (Pittman et al., 2008), *Tg(-17.6isl2b:tagRFP)*^{zc80}, *Tg(hsp70l:GFP)*^{mik6} and *Tg(hsp70l:cxcl12a-2A-EGFP)*^{zc85}. The transgenic line *Tg(hsp70l:cxcl12a-2A-EGFP)*^{zc85} was generated using the Tol2 kit (Kwan et al., 2007). The *cxcl12a* full-length middle clone was generated using primers with attB1R and attB2R recognition sites flanking the *cxcl12a* coding sequence, omitting the forward stop codon.

In situ hybridization

Embryos were dechorionated and fixed in 4% paraformaldehyde (PFA) overnight at 4°C, washed in PBS (prepared with DEPC-H_2O), dehydrated through a methanol series, and stored at -20°C. Whole-mount *in situ* hybridization staining was performed according to Thisse and Thisse (2008). For sectioning, embryos were prepared as

described previously (Pittman et al., 2008) and sectioned at 15 μm on a Reichert-Jung 2050 Supercut microtome with a glass knife. Images were taken on an Olympus BX51WI compound microscope using a SPOT RT3 camera. Images were processed using Adobe Photoshop CS2 software.

Immunohistochemistry

For whole-mount immunohistochemistry, embryos were fixed in 4% PFA overnight, washed in PBS, dehydrated through a methanol series and stored at -20°C, rehydrated and washed in PBST (PBS with 0.1% Tween-20), permeabilized with 0.1% collagenase for 15 min at room temperature (RT) (10 min for *shha, smo* and SANT75-treated embryos). Embryos were blocked for 2 hrs at RT with 10% newborn calf serum with 0.1% Tween-20 (NCST) and incubated in primary antibodies (overnight, 4°C). Embryos were washed in PBST and incubated 4 hrs in secondary antibodies plus ToPro3 (1:1000, Molecular Probes) at RT and washed in PBST. Primary antibodies used: mouse anti-GFP (1:200; Millipore), rabbit anti-GFP (1:200; Invitrogen), mouse anti-tagRFP (1:200; Evrogen), rabbit anti-Pax2a (1:300, gift of Dr. A. Picker); secondary antibodies used: goat anti-mouse 488 (1:200; Molecular Probes), goat anti-rabbit 488 (1:200; Invitrogen), goat anti-mouse cy3 (1:200; Jackson ImmunoResearch), goat anti-rabbit cy3 (1:200; Jackson ImmunoResearch).

Confocal microscopy

Antibody stained embryos were cleared in 50% glycerol/H_2O for 3 hrs at 4°C and stored at 4°C in 80% glycerol/H_2O. Heads were dissected and embedded between two

cover slips #0 separated by 2 layers of black electrical tape. Images were taken on a FV1000-XY Olympus IX81 confocal microscope using a 40x water objective. Maximum-intensity projection images were generated using ImageJ (Rasband, W.S., NIH).

SANT75 treatment

20 *isl2b:GFP* embryos were incubated in 40 μM SANT75 (gift of Dr. Shuo Lin, UCLA) in 1% DMSO in E3 or E2/GN (E2 + 10 μg/ml gentamycin sulfate) bath-applied in 6-well plates. For the 1-24 hpf treatment, SANT75 was removed by washing with E3 3x15 min. Intraretinal axon guidance phenotypes were scored using an Olympus SZX16 fluorescent stereomicroscope. Embryos with stalled RGC wave progression and no visible axon outgrowth were not included in the analysis.

Cell transplants

Embryos were anesthetized with tricaine, mounted in 1% low-melt agarose and covered with fish ringer. For RGC transplants: *isl2b:GFP* and *isl2b:tagRFP* transgenic embryos were used as donors and hosts. Roughly 20 RGC precursors were transplanted at 24 hpf. For ectopic *cxcl12a* expression: *hsp70l:EGFP* or *hsp70l:cxcl12a-2A-EGFP* embryos were used as donors. *shha* or wt embryos were used as hosts. About 50 anterior retinal cells were transplanted at 24 hpf into the anterior eye of host embryos. Hosts and donors were subjected to three rounds of heat-shock at 28, 32, and 36 hpf at 40ºC. For all transplants, embryos were fixed overnight at 4°C in 4% PFA, washed in PBS, dehydrated through a methanol series and stored at -20°C until processed for immunohistochemistry.

Morpholino injections

4 ng *ath5*MO was injected at the 1-cell stage as described previously (Pittman et al., 2008). Successful inhibition of RGC differentiation after *ath5*MO injection through 54 hpf was monitored using *isl2b:GFP* embryos, which also express GFP in the trigeminal ganglion. MO injected embryos were used as host embryos for cell transplant experiments as described above.

Statistical analysis

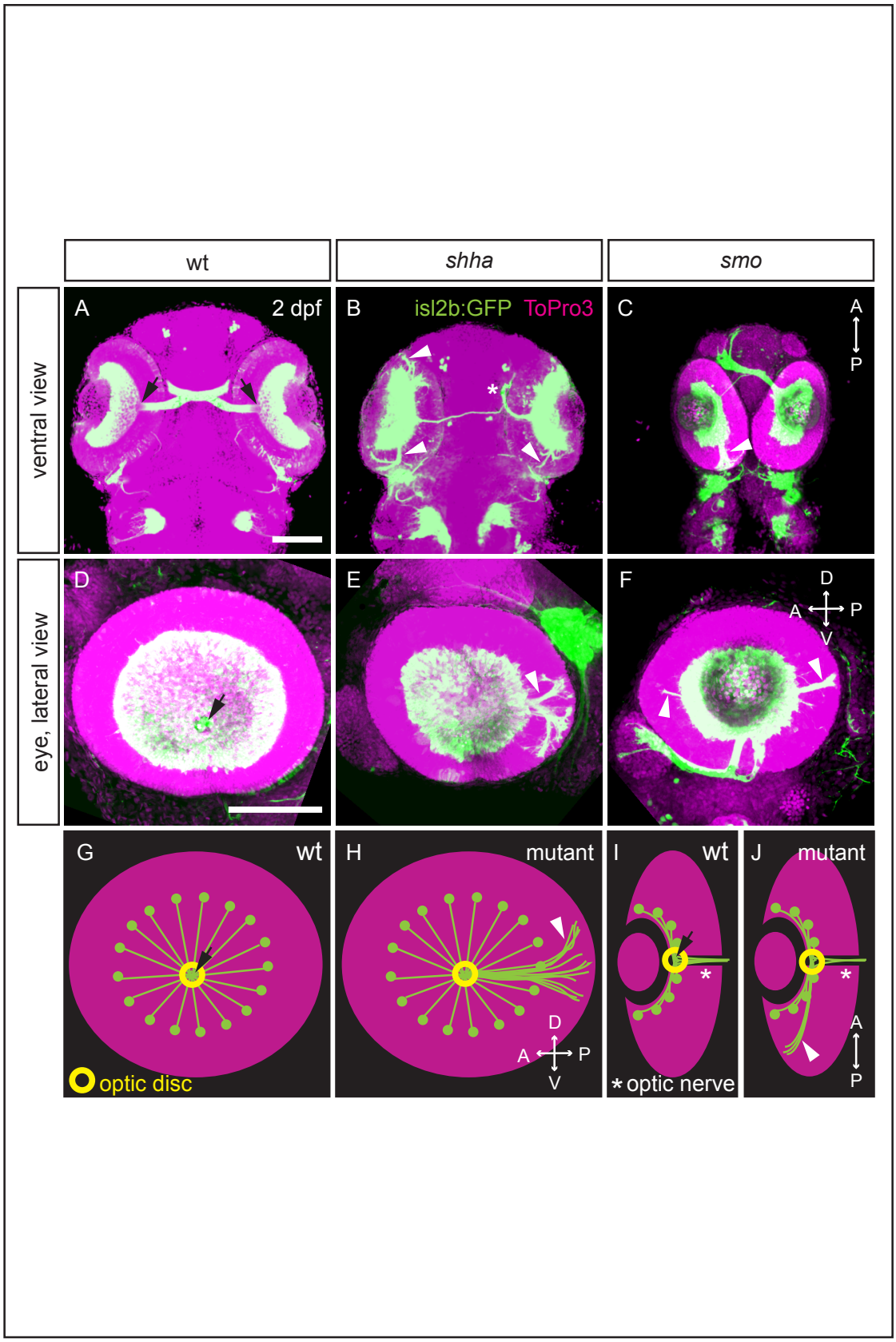
Fisher's exact test was calculated using http://faculty.vassar.edu/lowry/fisher.html website. Student t-test was calculated using Microsoft Excel. Mann-Whitney U: Embryos from three experiments were pooled for each genotype, ranked into categories. The percentage of embryos with delayed or no RGC layer differentiation was equal for both phenotypes, therefore these embryos were not included in the final analysis. Embryos were scored at 2 dpf using an Olympus SZX16 stereomicroscope. Statistical difference between the ranking of the two genotypes was calculated using Mann-Whitney U statistics on http://faculty.vassar.edu/lowry/utest.html website.

Results

Hh mutant RGC axons make intraretinal pathfinding errors

In wildtype (wt) zebrafish, RGC axons project towards the optic disc in the center of the retina and exit the eye through the optic nerve (Figure 4.1A, D, G, I; optic disc, *arrows*; optic nerve, *asterisk* in I). In mutants for *sonic hedgehog a* (*shha*), many RGC

Figure 4.1: Intraretinal axon pathfinding defects in mutants of the Hh pathway. Retinal projections at 2 days postfertilization (dpf) in wt, *shha* and *smo* embryos with *isl2b:GFP* (green) or *isl2b:tagRFP* (pseudocolored green) transgene; nuclei, ToPro3 (magenta). Ventral (A-C) or lateral views (D-F) of maximum-intensity confocal projections. Schematics of wt and mutant axon projections showing lateral (G, H) and ventral views (I, J). In wt embryos (A, D, G, I), RGC axons converge at the optic disc (*arrow*), where they turn and pass through the optic nerve (I, *asterisk*). In *shha* (B, E, H, J) and *smo* (C, F, H, J) mutants, some axons fail to exit the eye, projecting posteriorly or occasionally anteriorly within the eye (*arrowheads*). Hh mutants also exhibit misprojections to the ipsilateral optic tectum (*asterisk* in B). Scale bar 100 μm. D, dorsal; V, ventral; A, anterior; P, posterior.


wt
shha
smo
ventral view
eye, lateral view
A
2 dpf
B
isl2b:GFP ToPro3
C
A
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D
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D
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V
G
wt
optic disc
H
mutant
D
A
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wt
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mutant
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axons fail to exit through the optic nerve and become trapped within the eye, projecting posteriorly or sometimes anteriorly within the retina (Figure 4.1B, E, H, J, *arrowheads*). In addition, RGC axons that exit the eye through the optic nerve in *shha* mutants often make mistakes further along the pathway and grow dorsally to project to the ipsilateral optic tectum instead of crossing at the chiasm (Figure 4.1B, *asterisk*).

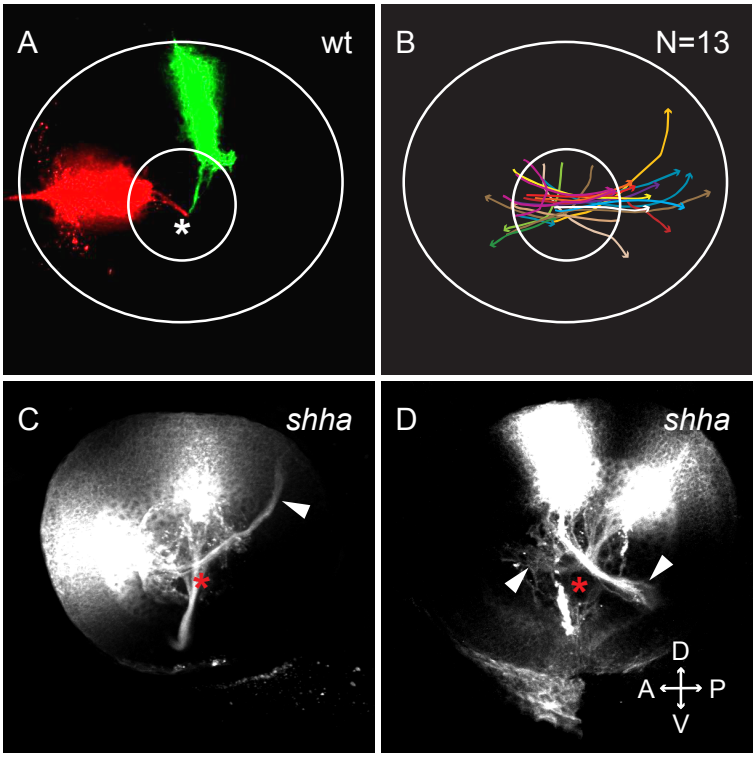
Zebrafish carry two paralogs of *shh*, *shha* and *shhb*. However, while both variants are expressed in the RGC layer (Neumann and Nuesslein-Volhard, 2000), only the loss of *shha* result in intraretinal axon pathfinding errors. Knockdown of *shhb* in wt embryos using a translation blocking morpholino (MO) did not induce intraretinal axon guidance errors, while injections into *shha* embryos led to severe midline defects similar to *smo* mutants (Varga et al., 2001), without an increase in the severity of the intraretinal pathfinding phenotype (data not shown).

Focal lipophilic dye injections in the RGC layer revealed that RGC axons from all quadrants of the *shha* retina project towards the optic disc, where some axons fail to turn and misproject within the eye, with a predominance of posterior over anterior projections (Figure 4.2).

The ligand Shh binds to Patched (Ptch) G-protein coupled transmembrane receptors, which signal through a second transmembrane protein, Smoothened (Smo), to activate the Hh signaling pathway (Ingham and McMahon, 2001). Zebrafish carry one *smo* gene and mutations in *smo* therefore lead to complete inhibition of the Hh signaling pathway. *Smo* mutant zebrafish embryos exhibit intraretinal axon guidance errors and ipsilateral projections analogous to the ones seen in *shha* mutants (Figure 4.1C, F, H, J). *Smo* embryos show additional strong midline patterning defects, but are rescued from

Figure 4.2: Focal dye injections show prevalence for posterior RGC axon projections in *shha* mutants.

Maximum-intensity confocal projections of lateral views of focal lipophilic dye injections into the retina of 2 dpf wt and *shha* embryos. (A) Wt eye injected with DiI anterior and DiO dorsal shows axon projections towards the optic disc (white *asterisk*), where the axons leave the eye through the optic nerve. (B) Diagram showing *shha* RGC axon projections as seen after DiI injections of 13 embryos. Axon bundles from all quadrant of the retina project towards the optic disc but fail to leave through the optic nerve and instead continue growing inside the eye. Posterior RGC projections are seen more commonly than anterior projections. (C, D) Examples of *shha* eyes focally injected with DiI. Misprojecting RGC axon bundles grow towards the optic disc (*red asterisk*) before projecting posteriorly or anteriorly (*arrowheads*). D, dorsal; V, ventral; A, anterior; P, posterior.


A
wt
B
N=13
C
shha
D
shha
D
A
P
V

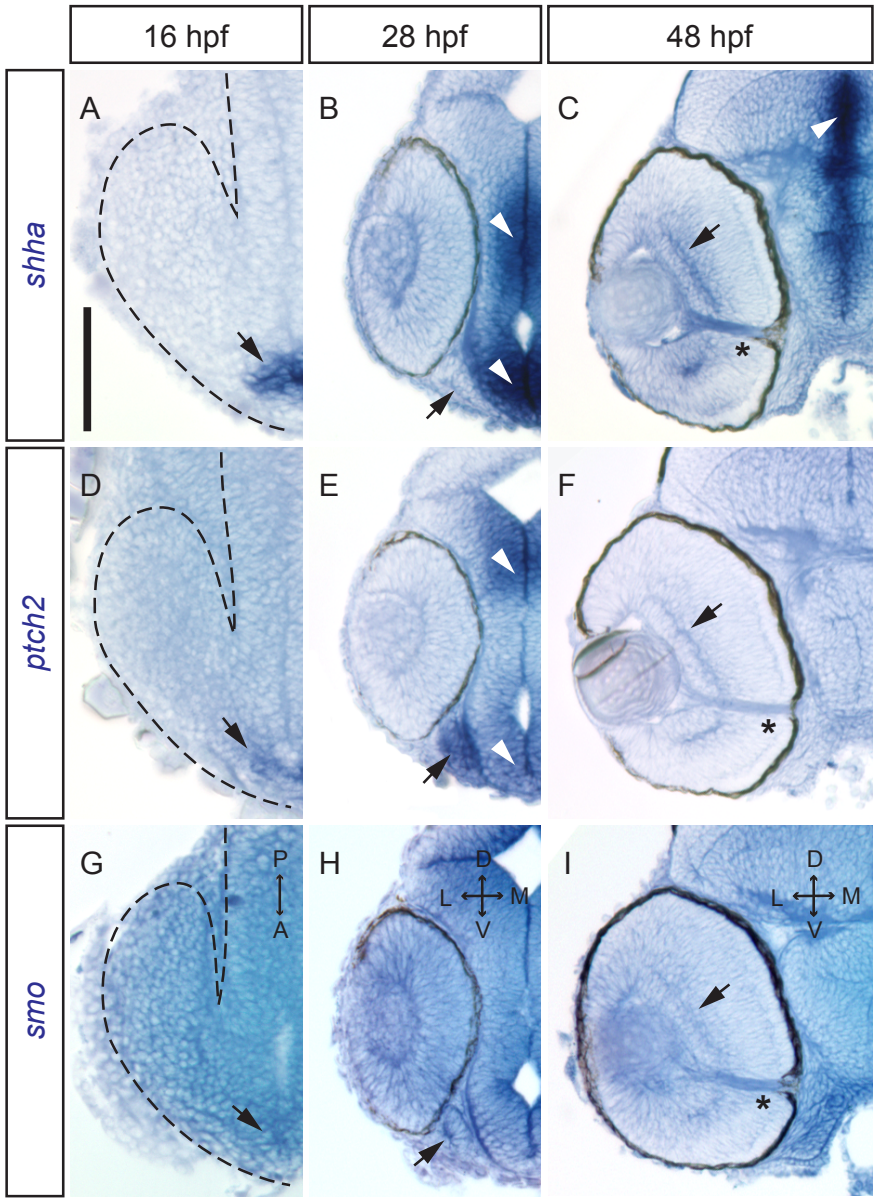
complete cyclopia by maternally expressed *smo* (Varga et al., 2001). In summary, Hh signaling is required for correct intraretinal axon pathfinding in zebrafish.

Hh pathway components are expressed during eye patterning and RGC axon outgrowth

To determine how Hh signaling regulates intraretinal axon pathfinding, we analyzed the spatial and temporal expression patterns of both ligand and receptor components during eye development and RGC axon outgrowth. We performed *in situ* hybridization for *shha, ptch2*, and *smo* at 16 hpf, after the optic vesicle has formed, at 28 hpf, just as RGCs start to differentiate, and at 48 hpf, when most RGCs have differentiated and several axons have reached the optic tectum. At 16 hpf, *shha*, *ptch2*, and *smo* mRNAs are expressed in the anterior midline neurectoderm (Figure 4.3A, D, G). While the expression of *shha* and *ptch2* is very specific, *smo* shows a broader expression throughout the head region. At 28 hpf, *shha* is strongly expressed at the midline (Figure 4.3B), while *ptch2* is expressed at the midline and also strongly in the optic stalk region (Figure 4.3E) and *smo* shows broad expression throughout the brain and the optic stalk (Figure 4.3H). At 48 hpf, *shha* midline expression is still strong (Figure 4.3C). In addition, all three Hh pathway genes analyzed are expressed in RGCs at this time (Figure 4.3C, F, I). Thus, Hh pathway components are expressed both during ocular tissue patterning, as well as during the period of RGC axon outgrowth, consistent with a potential role in both processes.

Figure 4.3: Hh pathway genes are expressed in the optic stalk and RGC layer.

In situ hybridizations for *shha*, *ptch2*, and *smo* mRNA. (A) 16 hpf, *shha* expression in anterior midline neurectoderm (*arrow*). (B) 28 hpf, *shha* expression at the midline (*arrowhead*) but not optic stalk (*arrow*). (C) 48 hpf, *shha* strongly expressed at the midline (*arrowhead*) and in RGC layer (*arrow*). mRNA also detected in the optic nerve (*asterisk*). (D) 16 hpf, *ptch2* expressed at the anterior midline (*arrow*). (E) 28 hpf, *ptch2* strongly expressed in the optic stalk (*arrow*) and midline (*arrowheads*). (F) 48 hpf, *ptch2* detected in the RGC layer (*arrow*) and optic nerve (*asterisk*). (G) 16 hpf, *smo* expressed throughout head region. (H) 28 hpf, *smo* broadly expressed, including optic stalk (*arrow*). (I) 48 hpf, *smo* localized in the RGC layer (*arrow*) and optic nerve (*asterisk*). 15 μm sections of whole mount *in situ* hybridizations. (A,D,G) dorsal views; A, anterior; P, posterior; (B,C,E,F,H,I) frontal views; D, dorsal; V, ventral; L, lateral; M, medial. Scale bar 100 μm.


16 hpf
28 hpf
48 hpf
shha
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A
B
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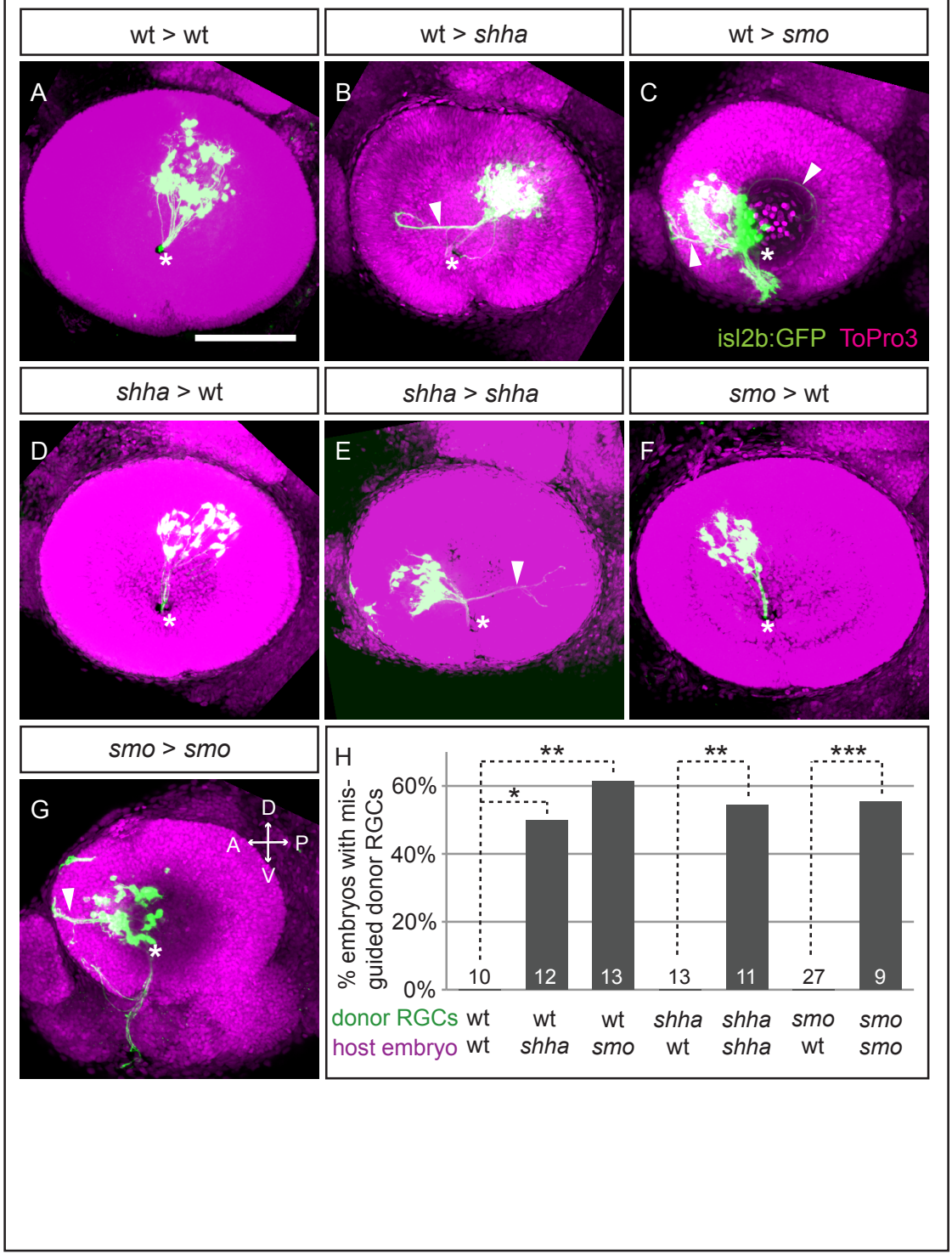
Shha and Smo act non-cell-autonomously in RGC axon pathfinding

With Hh pathway gene expression supporting either an indirect or a direct role in RGC axon pathfinding, we used cell transplants to functionally test for cell autonomy of Hh pathway components. If Hh signaling directly regulates the guidance of RGC axons, we would expect Shha to act non-cell-autonomously, while the receptor components would act cell autonomously in RGCs. On the other hand, if Hh signaling patterns the eye and optic stalk to ensure the correct cellular environment for intraretinal RGC axon pathfinding, we would expect both ligand and receptor to act non-cell-autonomously in RGC axon pathfinding.

We used wt, *shha,* and *smo* embryos to transplant RGC precursor cells at 24 hpf from donor into host embryos and analyzed donor RGC axon projections at 54 hpf. To visualize RGC projections, donor embryos carried the *isl2b:GFP* transgene, which labels most RGCs as well as neurons outside of the eye, including the trigeminal ganglion posterior to the eye (Pittman et al., 2008). When wt RGC precursor cells were transplanted into wt hosts all donor RGC axons exited the host eye through the optic nerve in 100% of the transplants (Figure 4.3A). However, when wt cells were transplanted into *shha* hosts, we observed misguided donor RGC axons in 50% of the transplants (Figure 4.4B, H (p=0.015)). Similarly, when wt cells were transplanted into *smo* hosts, misguided donor RGC axons were found in 62% of the mutant hosts (Figure 4.4C, H (p=0.006)). When *shha* RGCs were transplanted into wt hosts all donor RGC axons exited the eye normally (Figure 4.4D, H), while we observed misguided axons in 55% of the transplants when *shha* cells were transplanted into *shha* hosts (Figure 4.4E, H (p=0.003)). Similarly, donor axons from *smo* into wt transplants always exited the eye

Figure 4.4: RGC transplants demonstrate non-cell-autonomous functions of Shh and Smo in intraretinal axon pathfinding.

(A-G) Representative images of host eyes at 54 hpf after cell transplants at 24hpf. Lateral views of maximum-intensity confocal projections. Wt RGCs send out axons that exit the eye through the optic disc (*asterisk*) in wt hosts (A), but often misproject (*arrowheads*) in *shha* (B) and *smo* (C) hosts. *Shha* RGC axons always exit the eye in wt hosts (D), while many misproject in *shha* hosts (E). Similar results found with *smo* RGCs in wt (F) or *smo* (G) hosts. (H) Percentage of embryos with misrouted donor RGC axons. Numbers of embryos shown in base of bars. *p<0.05, **<0.01, ***<0.001, Fisher's exact test. Transplanted RGCs are *isl2b:GFP* or *isl2b:tagRFP* (pseudocolored green); nuclei, ToPro3 (magenta). Scale bar 100 μm. D, dorsal; V, ventral; A, anterior; P, posterior.


wt > wt
wt > shha
wt > smo
A
B
C
isl2b:GFP ToPro3
shha > wt
shha > shha
smo > wt
D
E
F
smo > smo
G
D
A
P
V
H
% embryos with mis-guided donor RGCs
60%
40%
20%
0%
**
*
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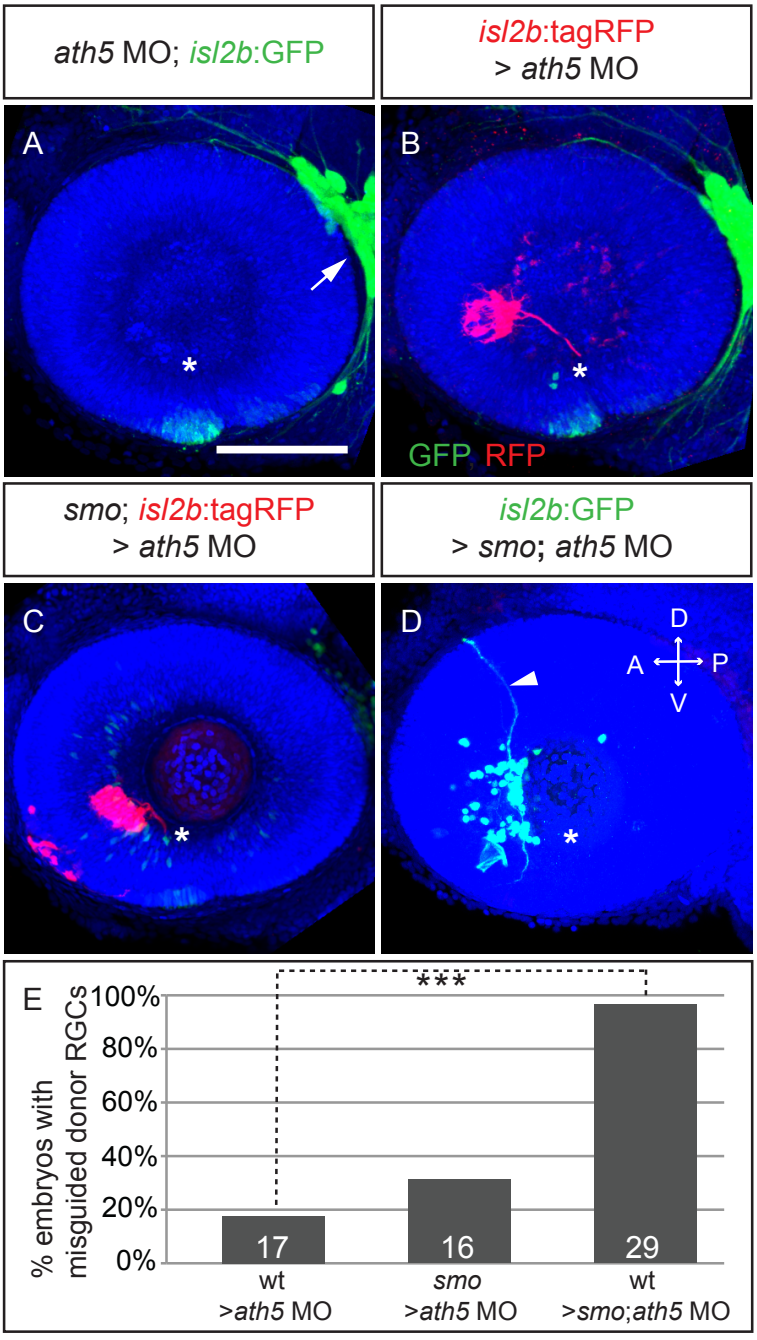
***
10
12
13
13
11
27
9
donor RGCs wt wt wt shha shha smo smo
host embryo wt shha smo wt shha wt smo

through the optic nerve (Figure 4.4G, H), while *smo* into *smo* transplants resulted in 56% of the transplants with misguided donor RGC axons (Figure 4.4G, H (p=0.003)). Altogether, we find that wt and mutant RGC axons are equally able to exit the eye in wt hosts but undergo pathfinding errors when transplanted into *shha* or *smo* mutant embryos. This indicates that both Shha and Smo act non-cell-autonomously in intraretinal axon pathfinding.

Previously, we found that pioneer RGCs are necessary to guide later born axons out of the eye (Pittman et al., 2008). Thus, although the non-cell-autonomous effect of Smo strongly suggests a role in eye patterning, this result could also be explained by axon-axon interactions, where donor RGC axons simply follow host pioneers, thereby masking a cell autonomous effect of Smo in intraretinal axon guidance. To prevent such pioneer-follower axon-axon interactions, we inhibited RGC differentiation in host embryos until at least 54 hpf with *ath5* MO injections. By transplanting donor RGCs into *ath5* morphants, we could analyze axon pathfinding of donor RGCs in a host RGC-free environment. *Isl2b:GFP* embryos were injected with 4 ng *ath5* MO at the 1-cell stage. *Isl2b:tagRFP* donor RGC precursor cells were transplanted at 24 hpf into *ath5* morphants and RGC axon projections were analyzed at 54 hpf. *Isl2b:GFP* expression in the trigeminal ganglion was used as a positive control to ensure successful inhibition of RGC differentiation in transgenic embryos (Figure 4.5A, *arrow*). When wt RGC precursor cells were transplanted into *ath5* morphants, donor RGC axons exited the eye in most cases; only 18% of the transplants showed intraretinal axon pathfinding errors (Figure 4.5B, E). Similarly, *smo* donor axons exited the eye through the optic nerve in most *ath5* morphants; 31% of the transplants exhibited errors (Figure 4.5C, E (p=0.44)). On the

Figure 4.5: Transplants into RGC-free hosts confirm non-cell-autonomous effect of Smo in intraretinal axon pathfinding.

Maximum-intensity confocal projections of 54 hpf *isl2b:GFP* or *isl2b:tagRFP* embryos injected with *ath5* MO at 1-cell stage (A) and transplanted with donor cells at 24 hpf (B-D). (A) No RGC differentiation after *ath5* MO; trigeminal ganglion as control for transgene expression (*arrow*). (B) Wt RGC axons (*isl2b:tagRFP*, red) in *isl2b:GFP* (green) *ath5* morphants rarely make errors. (C) *smo* (*isl2b:tagRFP*) RGC axons in *isl2b:GFP ath5* morphants are rarely misguided. (D) In contrast, wt (*isl2b:GFP*) RGC axons in *smo ath5* morphants make errors (*arrowhead*) in almost 100% of transplants. (E) Percentage embryos with misrouted axons. Numbers of embryos shown in base of bars. ***$p<0.005$, Fisher's exact test. Nuclei, ToPro3 (blue); optic disc, *asterisk*. Scale bar, 100 μm. D, dorsal; V, ventral; A, anterior; P, posterior.


ath5 MO; isl2b:GFP
isl2b:tagRFP
> ath5 MO
A
B
GFP RFP
smo; isl2b:tagRFP
> ath5 MO
isl2b:GFP
> smo; ath5 MO
C
D
D
A
P
V
E
% embryos with misguided donor RGCs
100%
80%
60%
40%
20%
0%
***
17
16
29
wt
>ath5 MO
smo
>ath5 MO
wt
>smo;ath5 MO

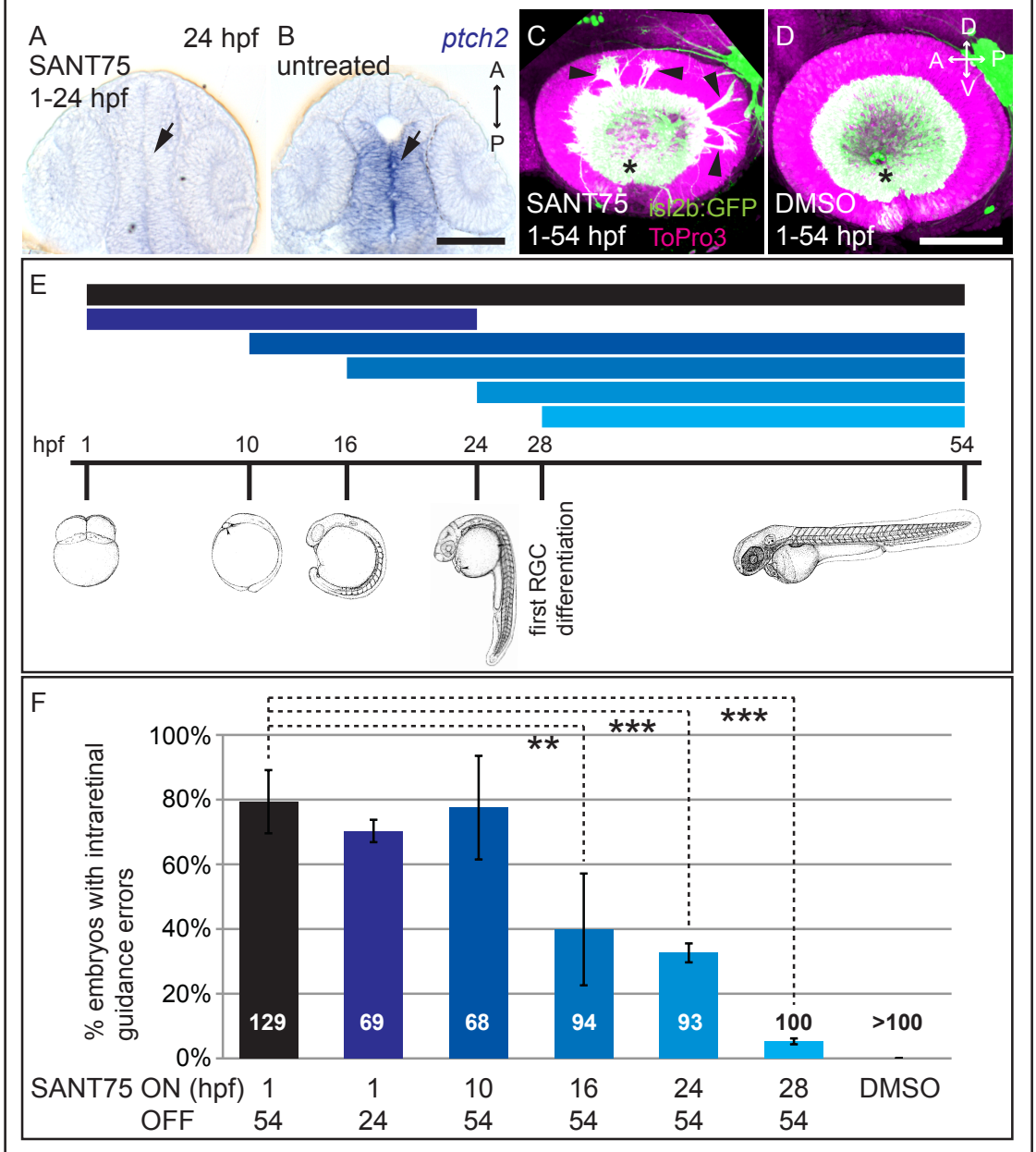
other hand, wt donor RGC axons were misrouted in *smo*;*ath5* MO host eyes in 96% of the transplants (Figure 4.5D, E (p=3.9E-8)). In summary, our results demonstrate that Smo is required in the environment to ensure correct RGC axon pathfinding, but not in RGCs themselves.

Hh signaling is not required during axon pathfinding
for correct axon outgrowth

Determining the temporal requirement of Hh pathway activation for correct intraretinal axon pathfinding can provide additional evidence for a role for Hh signaling in either axon guidance or tissue patterning. To determine the time at which Hh signaling is necessary for intraretinal axon pathfinding, we used pharmacological treatment to inhibit Hh signaling during embryonic development. We applied the small molecule compound SANT75 (Smoothened antagonist 75; kind gift of Shuo Lin), which specifically inhibits Smo (Yang et al., 2009). We chose SANT75 rather than the more common cyclopamine because of better solubility and stability properties. Because *ptch* receptors and *gli* transcription factors (downstream of *smo*) are themselves target genes of the Hh pathway, expression levels of *ptch2* and *gli1* can be used as readouts for pathway inhibition. SANT75 treatment has been shown to inhibit *ptch2* and *gli1* expression in a dose-dependent manner (Yang et al., 2009). 40 μM SANT75 application resulted in downregulation of *ptch2* expression in the brain at 24 hpf (Figure 4.6A, B, *arrowheads*) but no cyclopia. Bath application of 40 μM SANT75 from 1-54 hpf induced a strong intraretinal axon pathfinding phenotype in 79% of embryos carrying the *isl2b:GFP*

Figure 4.6: Temporal inhibition of Hh signaling shows that Shh is required during eye patterning.

40 μM SANT75 was bath applied to inhibit Hh signaling for specific time periods during embryonic development. Maximum-intensity confocal projections of lateral views (54 hpf). (A) 1% DMSO does not affect RGC axon projections in *isl2b:GFP* embryos (green), while (B) 40 μM SANT75 (1-54 hpf) yields misguided RGC axons (*arrowheads*). Nuclei, ToPro3 (magenta); optic disc, *asterisk*. Scale bar, 100 μm. D, dorsal; V, ventral; A, anterior; P, posterior. (C) *ptch2* mRNA expressed at the midline (*arrow*) in DMSO treated embryos (24 hpf), while (D) expression is lost (*arrow*) after SANT75 treatment (1-24 hpf). 15 μm sections of whole mount *in situ* hybridizations, dorsal views. Scale bars 100 μm. A, anterior; P, posterior. Different SANT75 application time-points (E) and percentage embryos with intraretinal guidance errors thereafter (F). Total number of embryos shown in base of bars. Error bars, S.D. n≥3 experiments, $^{**}p<0.01$, $^{***}<0.001$, Student's t-test.


A
SANT75
1-24 hpf
24 hpf
B
untreated
ptch2
A
P
C
SANT75
1-54 hpf
Isl2b:GFP
ToPro3
D
DMSO
1-54 hpf
D
A
P
V
E
hpf 1
10
16
24
28
54
first RGC differentiation
F
% embryos with intraretinal guidance errors
100%
80%
60%
40%
20%
0%
**
***
***
129
69
68
94
93
100
>100
SANT75 ON (hpf) 1 1 10 16 24 28 DMSO
OFF 54 24 54 54 54 54

transgene (Figure 4.6C, F), while DMSO control embryos never showed pathfinding errors (Figure 4.6D, F).

We next sought to determine the temporal requirement of Hh signaling for intraretinal axon pathfinding. At 10 hpf, the eye field is specified and the optic vesicle is just forming. At 16 hpf, the optic vesicle is formed but eye patterning is still ongoing. At 24 hpf, basic eye patterning is completed and at 28 hpf, the first RGCs start to differentiate. Starting SANT75 treatment at these specific timepoints allowed us to determine whether Hh signaling is required before optic vesicle specification (1-10 hpf), for optic vesicle specification and basic eye patterning (10-24 hpf), or during the time of axon outgrowth (after 28 hpf). Treatment with 40 μM SANT75 from 10-54 hpf resulted in 78% of *isl2b:GFP* embryos with intraretinal axon pathfinding errors (Figure 4.6F). Similarly, when we started treatment at 1 hpf and washed out SANT75 at 24 hpf, we found that 70% of the treated *isl2b:GFP* embryos had intraretinal axon pathfinding errors (Figure 4.6F). These results are not significantly different from continuous treatment (1-54 hpf), where 79% of the embryos showed errors. Starting treatment at 16 hpf or 24 hpf, on the other hand, resulted in significantly fewer embryos with pathfinding errors, with 40% (p=0.002) and 33% (p=0.0001) of the embryos with errors, respectively (Figure 4.6F). When treatment began at 28 hpf, just as the first RGCs start to differentiate, only 5% of the embryos showed intraretinal pathfinding errors (Figure 4.6F; p=4.4E-6). The strong decrease of axon pathfinding phenotype with treatment starting at 24 and 28 hpf indicates that Hh signaling is not necessary at the time of RGC axon pathfinding out of the eye. In addition, our wash-off experiment showed that early inhibition of Hh signaling causes intraretinal axon pathfinding errors similar to those seen with continuous

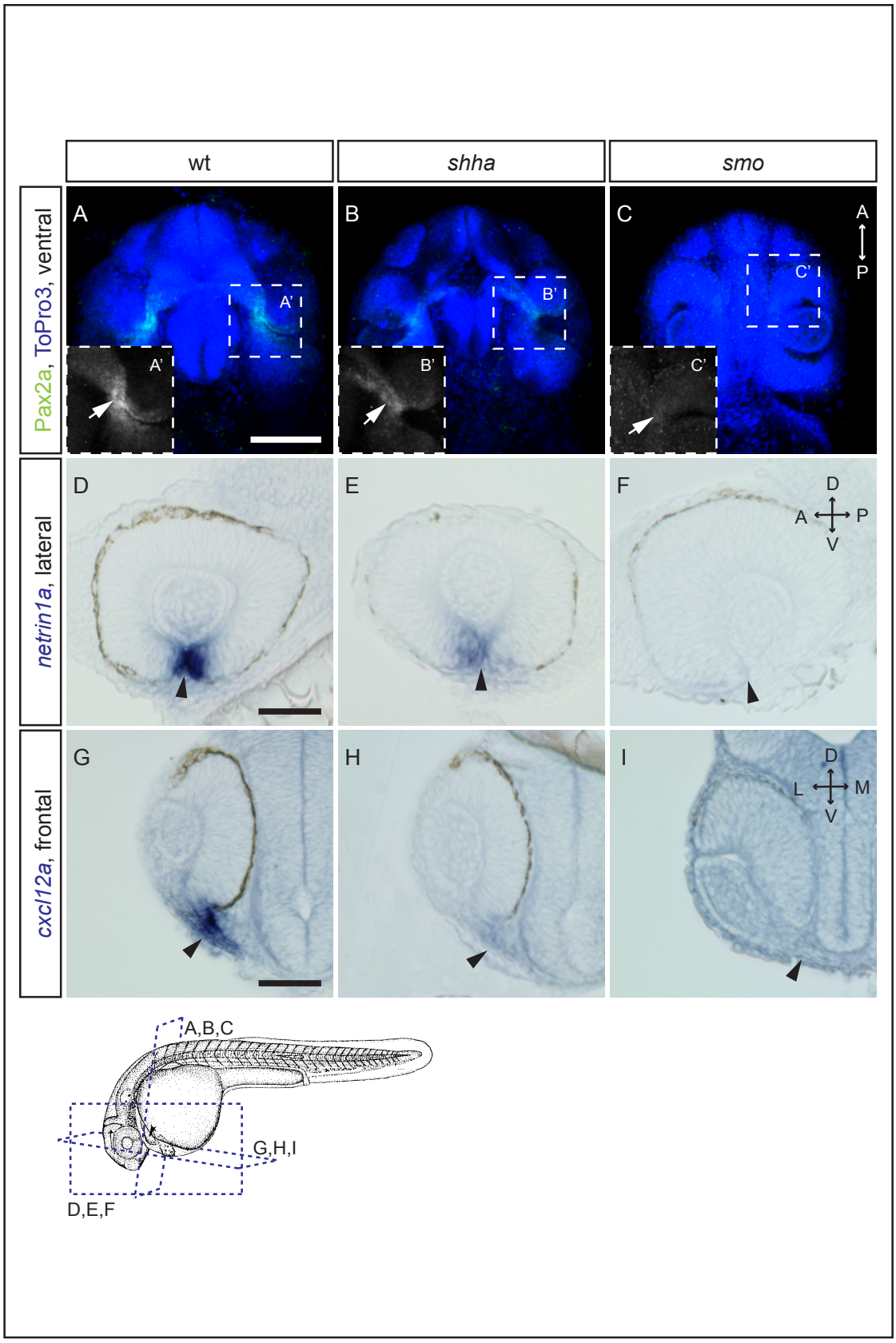
treatment. In summary, our results show that inhibition of Hh signaling during optic vesicle patterning is sufficient to induce intraretinal axon pathfinding errors later during development and therefore argue against a direct role of Shh in intraretinal axon guidance.

Optic stalk markers are downregulated in Hh pathway mutants

The strong indication for a role of Hh signaling in eye patterning but not direct RGC axon guidance prompted us to analyze the expression of several optic stalk markers in *shha* and *smo* mutants. Pax2 is a transcription factor expressed in the developing optic stalk (Macdonald et al., 1995). Immunohistochemistry at 28 hpf showed a downregulation of Pax2a expression in *shha* embryos compared to wt, and no Pax2a expression in the optic stalk in *smo* embryos (Figure 4.7A-C). *netrin1a*, which encodes a known axon guidance molecule, is expressed along the optic fissure in the developing zebrafish eye (Macdonald et al., 1997). *In situ* hybridization at 28 hpf revealed that *netrin1a* expression is decreased in *shha* mutants and no expression is found in *smo* eyes (Figure 4.7D-F). *chemokine ligand 12a* (*cxcl12a*) and its homologue *cxcl12b* are expressed in the optic stalk and Cxcl12b was proposed to have an attractive effect on RGC axons inside the eye in zebrafish (Li et al., 2005). Again, *in situ* hybridization at 28 hpf showed a strong downregulation of *cxcl12a* in the optic stalk in *shha* embryos and no expression in the optic stalk in *smo* mutants (Figure 4.7G-I). Thus, loss of Hh signaling leads to a downregulation of known transcription factors and axon guidance molecules in the stalk region.

Figure 4.7: Expression of optic stalk markers is decreased in Hh mutants.

(A-C) Maximum-intensity confocal projections of embryos stained for Pax2a (green) by immunohistochemistry (28 hpf); nuclei, ToPro3 (blue). Ventral views. A, anterior; P, posterior. Pax2a reduced in *shha* (B) and absent in *smo* (C) in optic stalk (arrow) compared to wt (A). (A'-C') Insets show magnified optic stalk region, Pax2a staining only. (D-F) *netrin1a* mRNA expression at the optic fissure (*arrowheads*). 15 μm sagittal sections of whole mount *in situ* hybridizations (28 hpf). D, dorsal; V, ventral; A, anterior; P, posterior. *netrin1a* at the optic fissure (arrowhead) decreased in *shha* (E) and lost in *smo* (F) compared to wt (D). (G-I) *cxcl12a* mRNA expression in the optic stalk (*arrowheads*). Coronal sections of whole mount *in situ* hybridizations (28 hpf). D, dorsal; V, ventral; L, lateral; M, medial. *cxcl12a* expression in optic stalk is reduced in *shha* (H) and lost in *smo* (I) compared to wt (G). Scale bars, 100 μm.


wt
shha
smo
Pax2a, ToPro3, ventral
netrin1a, lateral
cxcl12a, frontal
A
B
C
A'
B'
C'
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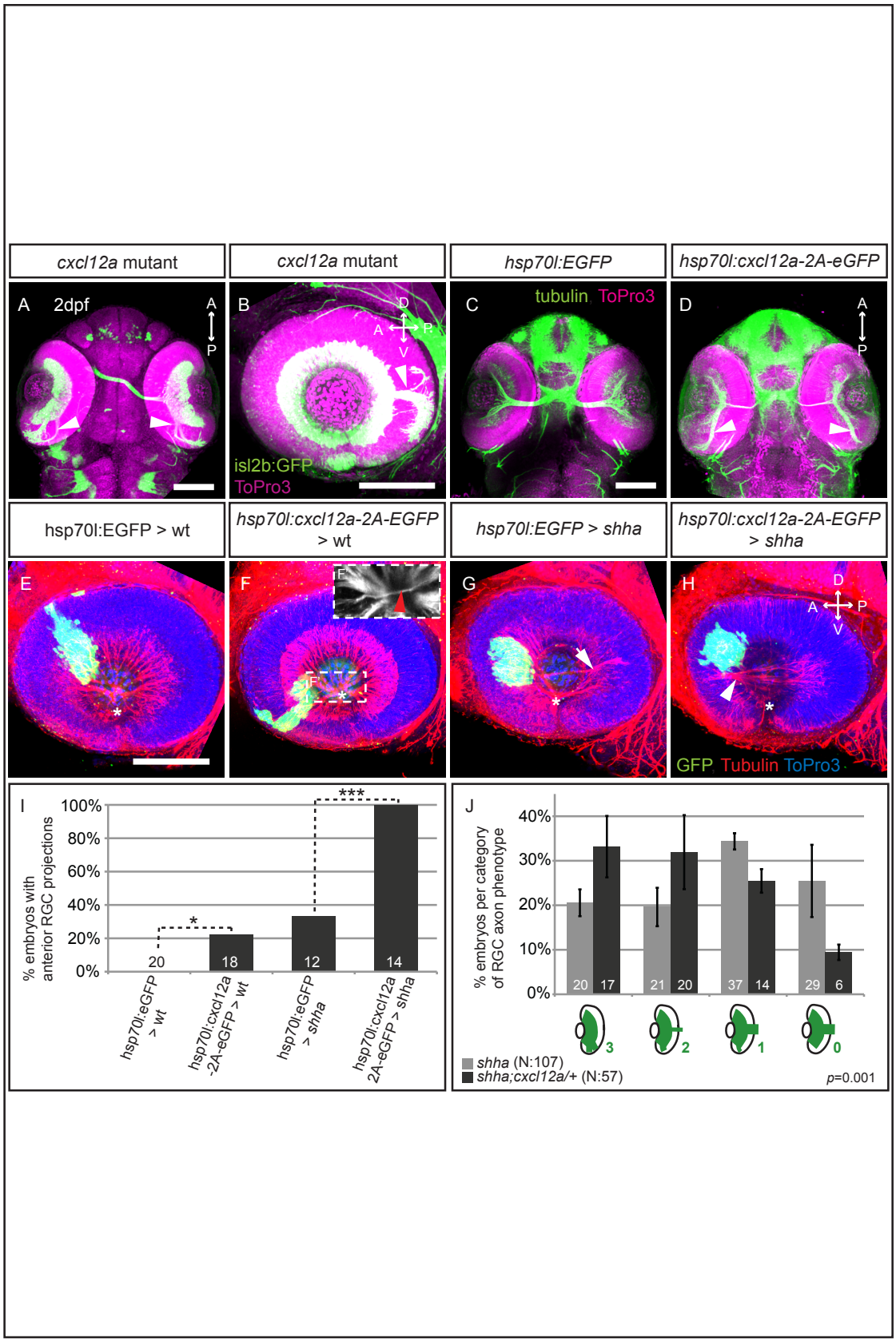
A,B,C
G,H,I
D,E,F

Cxcl12a has an attractive effect on *shha* RGC axons

The downregulation of optic stalk markers and specifically genes known to act as guidance molecules suggests a model in which Hh signaling during eye specification regulates optic stalk/disc expression of one or several guidance molecules, which are necessary for correct RGC axon pathfinding out of the eye. While *netrin1* mutants show intraretinal axon guidance errors in mouse (Deiner et al., 1997), we observed no errors using morpholino knockdown in zebrafish (data not shown). In contrast, zebrafish mutants for *chemokine receptor 4b* (*cxcr4b*), a receptor for *cxcl12a*, have been reported to exhibit intraretinal axon pathfinding errors (Li et al., 2005). Analysis of intraretinal RGC projections in *cxcl12a* mutants revealed the same pathfinding errors with a high penetrance (Figure 4.8A, B). The intraretinal axon guidance phenotypes in *cxcl12a* and *cxcr4b* mutants are strikingly similar to phenotypes found in Hh pathway mutants. Therefore, we tested whether downregulation of chemokine signaling at the optic disc in Hh pathway mutants may be responsible for their intraretinal axon pathfinding phenotype. It was reported that Cxcl12b has an attractive effect on RGC axons inside the eye in wt embryos (Li et al., 2005). To determine if Cxcl12a has a similar effect, we induced ectopic *cxcl12a* expression and analyzed whether RGC axons show attraction towards this chemokine. We made an expression construct with the heat-shock promoter driving full-length *cxcl12a* (*hsp70l:cxcl12a-2A-EGFP*). Global overexpression of Cxcl12a after three heat-shocks at 28, 32, and 36 hpf, respectively, led to intraretinal axon guidance errors similar to *cxcl12a* loss of function (Figure 4.8D). A control line expressing EGFP under the heat-shock promoter did not induce any axon guidance errors (Figure 4.8C).

Figure 4.8: Cxcl12a acts as a RGC axonal attractant in wt and shha and interacts genetically with the Hh pathway.

Maximum-intensity confocal projections of ventral (A, C, D) and lateral views (B, E-H) at 2 dpf. (A,B) *Cxcl12a* mutants exhibit intraretinal axon guidance errors (arrowheads). *Isl2b:GFP* (green); nuclei, ToPro3 (magenta). (C) Normal axonal projections in *hsp70l:EGFP* embryos after heatshock. (D) Global *Cxcl12a-2A-EGFP* overexpression induces intraretinal axon guidance errors (*arrowheads*). α-tubulin (pseudocolored green); nuclei, ToPro3 (magenta), EGFP not shown. (E-H) Cxcl12a expressing cells attract RGC axons in wt and *shha* embryos. Transplanted E*GFP* or *cxcl12a-2A-EGFP* expressing cells (green), α-tubulin (red); nuclei, ToPro3 (blue). (E) Anterior EGFP expressing cells in wt embryos do not affect RGC outgrowth. (F) Anterior projections in wt embryos with anterior Cxcl12a expressing cells. (F') Substack of boxed region in (F) with misguided axons (*red arrowhead*). (G) Rare anterior projections in *shha* embryos with EGFP expressing cells. (H) *Shha* embryos always show anterior projections with anterior Cxcl12a expressing cells. Optic disc, *asterisk*. Scale bars 100 μm. Ventral views, A, anterior; P, posterior. Lateral views, D, dorsal; V, ventral; A, anterior; P, posterior. (I) Percentage of host embryos with anterior RGC projections. Numbers of embryos shown in base of bars. *p<0.05, ***<0.001, Fisher's exact test. (J) Analysis of genetic interaction between *shha* and *cxcl12a*. Percentage of embryos per category (0-3) of RGC axon projection phenotype in *shha* (light grey) and *shha*;*cxcl12a*/+ (dark grey). Error bars, SEM, n=3 experiments. Mann-Whitney U test, p=0.00103, of embryos ranked in 4 categories in *shha* (N=107) and *shha*;*cxcl12a*/+ (N=57) populations.


cxcl12a mutant
cxcl12a mutant
hsp70l:EGFP
hsp70l:cxcl12a-2A-eGFP
A 2dpf
A
P
B
D
A
P
V
isl2b:GFP
ToPro3
C
tubulin ToPro3
D
A
P
hsp70l:EGFP > wt
hsp70l:cxcl12a-2A-EGFP > wt
hsp70l:EGFP > shha
hsp70l:cxcl12a-2A-EGFP > shha
E
F
G
H
D
A
P
V
GFP Tubulin ToPro3
I
% embryos with anterior RGC projections
100%
80%
60%
40%
20%
0%
*
***
20
18
12
14
hsp70l:eGFP > wt
hsp70l:cxcl12a -2A-eGFP > wt
hsp70l:eGFP > shha
hsp70l:cxcl12a 2A-eGFP > shha
J
% embryos per category of RGC axon phenotype
40%
30%
20%
10%
0%
20 17
21 20
37 14
29 6
3
2
1
0
shha (N:107)
shha;cxcl12a/+ (N:57)
p=0.001

To determine if Cxcl12a has an attractive effect on RGC axons, we transplanted retinal cells from *hsp70l:cxcl12a-2A-EGFP* embryos into the anterior eye of wt or *shha* hosts at 24 hpf and subjected the embryos to 3 rounds of heat-shock. At 54 hpf we scored host embryos for anterior RGC axon projection, as indication for an attractive effect of Cxcl12a. Control cells expressing EGFP only did not lead to any anterior RGC axon projections (Figure 4.8E, I), while 22% of wt embryos transplanted with Cxcl12a expressing cells showed anterior RGC projections (Figure 4.8F, I, p=0.04). In 33% of *shha* embryos with anteriorly placed EGFP expressing cells we observed anterior projections (Figure 4.8I), while posterior projections were more common in these embryos (Figure 4.8G, *arrow*). Here, anterior and posterior projections represent pathfinding errors due to the loss of *shha* and not due to an attractive effect of EGFP (Figure 4.1B, Figure 4.2). In sharp contrast, Cxcl12a expression in the anterior eye of *shha* mutant hosts led to anterior projections in 100% of the transplants (Figure 4.8H, I, p=0.0003). These results indicate that Cxcl12a has an attractive effect on RGC axons in both wt and *shha* embryos. In addition, ectopic Cxcl12a expression in *shha* mutant eyes resulted in a higher percentage of embryos exhibiting anterior projections than in wt eyes, possibly due to the decreased endogenous Cxcl12a at the optic disc in *shha* mutants.

The Hh and chemokine pathway interact genetically for intraretinal axon guidance

To further investigate the interaction between the Hh and chemokine pathways for intraretinal axon guidance, we analyzed the axon pathfinding phenotype in *shha* mutants which are either wt or heterozygous for *cxcl12a*. We crossed *shha*/+;*isl2b:GFP* fish to

shha/+;*cxcl12a*/+;*isl2b:GFP* carriers. At 2 days post fertilization, we scored the intraretinal pathfinding phenotypes and grouped the phenotypes into four categories, '0': no errors, all axons exit the eye; '1': few errors, most axons leave the eye; '2': most axons make errors inside the eye, few exit; '3': all axons make errors, no axons leave the eye (Figure 4.7J). All *shha* embryos were then genotyped. The *shha* and *shha;cxcl12a*/+ embryos were separated and the severity of the intraretinal axon pathfinding phenotype for both groups was analyzed using Mann-Whitney U statistics. Our analysis showed that the severity of the axon pathfinding phenotype was significantly increased in *shha*;*cxcl12a*/+ embryos compared to *shha* (p=0.001). Since *cxcl12a* heterozygous embryos do not exhibit any intraretinal axon guidance errors, our finding that the loss of one allele of *cxcl12a* increases the severity of intraretinal axon pathfinding errors in *shha* mutants indicates that the Hh and chemokine pathways interact genetically for the guidance of RGC axons inside the eye.

Discussion

Previous research has shown both the involvement of Shh in tissue patterning as well as in direct axon guidance, such in patterning of the spinal cord and guidance of commissural axons across and along the midline (Ericson et al., 1997; Briscoe and Ericson, 1999; Charron et al., 2003; Bourikas et al., 2005; Okada et al., 2006; Yam et al., 2009; Domanitskaya et al., 2010), as well as in optic stalk and retina patterning and RGC axon guidance along the retinotectal pathway (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003; Trousse et al., 2001; Kolpak et al., 2005, 2009; Sánchez-Camacho and Bovolenta, 2008; Fabre et al., 2010; Gordon et al., 2010). With zebrafish as a model

system, we were able to directly address the spatial and temporal requirement for Hh signaling for intraretinal axon pathfinding and determined that Hh signaling acts non-cell-autonomously for intraretinal axon pathfinding and that Hh pathway activity is required during early eye patterning for correct intraretinal axon pathfinding later in development. Additionally, we revealed a genetic interaction of the Hh and chemokine signaling pathway for intraretinal axon guidance and showed that Cxcl12a acts as an attractant for RGC axons inside the eye.

Shh regulates optic stalk patterning but not
direct RGC axon guidance in zebrafish

Shh expressed by notochord and floorplate cells forms a gradient along the proximodistal axis of the embryo, which is necessary for the specification of optic stalk and retina tissue in both Xenopus and zebrafish (Perron et al., 2003; Ekker et al., 1995; Macdonald et al., 1995). High levels of Shh induce the expression of the Pax2 transcription factor in proximal tissue, while distal optic tissue exposed to low levels of Shh expresses Pax6 (Ekker et al., 1995; Macdonald et al., 1995). Overexpression of Shh leads to an expansion of the Pax2 positive optic stalk region, while loss of midline structures and consequently loss of midline-derived Hh signaling results in an expansion of the Pax6 expressing retinal tissue (Ekker et al., 1995; Macdonald et al., 1995). In our study, we confirmed that loss of Hh signaling in *shha* and *smo* mutants leads to a downregulation of, or loss of Pax2 expression, respectively, in the zebrafish embryo.

Later in development, murine RGCs express Shh and protein is localized in axons and growth cones as they exit the eye, while neuroepithelial cells in the optic nerve

express the receptor Ptc (Wallace and Raff, 1999; Dakubo et al., 2003). This RGC-derived Hh activity was shown to be required for proliferation and maintenance of the astrocyte population in the optic nerve (Wallace and Raff, 1999; Dakubo et al., 2003, 2008). The role of RGC-derived Shh in regulation of astrocyte proliferation in the mouse optic nerve occurs after basic eye patterning has occurred and axons are leaving the eye. Although the exact timecourse of retinal glia development in zebrafish is unclear, we did not detect a role for Hh signaling in intraretinal pathfinding at a corresponding timepoint.

Hh pathway receptors are also expressed in murine RGCs and ganglion-cell-autonomous Hh signaling has been shown to regulate intraretinal axon guidance in mice (Sánchez-Camacho and Bovolenta, 2008). Similarly, inhibition of Hh signaling using cyclopamine in chicken suggested a role for Shh as a direct axon guidance molecule for intraretinal axon pathfinding (Kolpak et al., 2005). So while we detected expression of both Hh pathway ligand and receptors in the RGC layer at the time of axon outgrowth, we showed a RGC-non-autonomous role for Hh signaling in intraretinal axon pathfinding. Instead we observed a requirement for Hh pathway activity during early eye development for subsequent retinal pathfinding in zebrafish. The induction of intraretinal pathfinding errors with treatment starting during early optic vesicle formation in zebrafish is supported by a previous study, which used cyclopamine to inhibit the Hh signaling pathway (Kay et al., 2005). We propose that in zebrafish Hh signaling acts in patterning of the optic stalk early during eye development but not in direct axon guidance at the time of axon outgrowth to regulate correct intraretinal pathfinding in zebrafish. This role in patterning of the optic stalk corresponds well with previous findings showing that Hh signaling regulates axon pathfinding at the zebrafish midline indirectly by

determining glial cell position (Barresi et al., 2005). Therefore, although Hh signaling has a conserved role in vertebrate retinal axon pathfinding, the mechanisms appear to be different in different model systems.

Axon guidance at the optic disc

After reaching the optic disc, RGC axons need to turn into the optic nerve to exit the eye. Failure to turn leads to intraretinal pathfinding errors where axons overshoot the disc and project within the eye. In both mouse and zebrafish, Hh pathway mutants show intraretinal axon pathfinding errors (Sánchez-Camacho and Bovolenta, 2008; Schauerte et al., 1998). Additionally, mouse *netrin1* mutants also exhibit intraretinal axon guidance errors (Deiner et al., 1997). Intraretinal axon pathfinding errors in a conditional mouse model with loss of *shh* in RGCs has been explained by the lack of *netrin1* expression at the optic disc due to the loss of astrocyte precursor cells in this region (Dakubo et al., 2003). Similar to mouse *netrin1* (Deiner et al., 1999), zebrafish *netrin1a* is expressed in the optic disc/stalk region during RGC axon outgrowth (Park et al., 2005) and we show that loss of Hh signaling leads to downregulation of *netrin1a* expression in the stalk. But in zebrafish, we did not observe any retinal pathfinding errors using MO-mediated knockdown of *netrin1a*. None of the other zebrafish *netrin* paralogs are expressed in the eye during development (Park et al., 2005), thus making it unlikely that any of these genes could compensate for the loss of *netrin1a* in the optic stalk. We found that the chemokine *cxcl12a* is similarly transcriptionally regulated by Hh signaling. In addition, *cxcl12a* mutants and mutants for its receptor, *cxcr4b*, exhibit intraretinal axon pathfinding errors very similar to Hh mutants. Therefore it is likely that while *netrin-1* is the main

guidance cue inducing turning at the optic disc in mice, this role is taken by *cxcl12a* in zebrafish.

Chemokine signaling in axon guidance

A few studies have implicated chemokine signaling in axon guidance, demonstrating both attractive (Li et al., 2005; Arthur et al., 2009) and repulsive effects (Xiang et al., 2002) of Cxcl12 on axons, as well as a modulatory effect on other direct guidance cues (Chalasani et al., 2003, 2005; Lieberam et al., 2005). In zebrafish, *cxcl12a* is expressed in the distal optic stalk and MO-mediated knockdown of *cxcl12a* was shown to induce intraretinal axon pathfinding errors (Li et al., 2005). Additionally, we showed here that *cxcl12a* mutants exhibit a highly penetrant intraretinal axon pathfinding phenotype. *Cxcl12b* is localized in the proximal stalk region but knockdown of *cxcl12b* did not induce intraretinal pathfinding errors (Li et al., 2005). These findings show that *cxcl12a* is necessary for intraretinal axon pathfinding and demonstrate that *cxcl12b* cannot compensate for this role. Previously, it was shown that Cxcl12b misexpression in the eye has an attractive effect on RGC axons (Li et al., 2005). With *cxcl12a* being the required *cxcl12* paralog for pathfinding inside the eye, we showed that Cxcl12a exhibits a similar attractive effect on both wt and *shha* mutant RGC axons inside the eye. This result demonstrates that Shh is not required as a competence factor for the attractive effect of Cxcl12a. In contrast, we found that *shha* mutant axons showed a stronger attraction towards misexpressed Cxcl12a in the anterior eye compared to wt axons. This could be explained by the stark downregulation of *cxcl12a* in *shha* mutants in the optic stalk. While endogenous Cxcl12a at the optic disc attracts wt axons into the stalk, thereby

counteracting the effect of misexpressed Cxcl12a in the anterior eye, the low level of Cxcl12a at the optic disc in *shha* mutants could allow for a stronger attraction of RGC axons towards the misexpressed Cxcl12a.

Genetic interaction between the Hh and chemokine pathways

Hh signaling was shown to promote the expression of chemokine pathway components at the level of *cxcl12* and *cxcr4* in cholangiocyes, endothelial progenitor cells, and medulloblastoma (Omenetti et al., 2009; Yamazaki et al., 2008; Yoon et al., 2009). The relation between chemokine signaling and the Hh signaling pathway for axon pathfinding, on the other hand, has not been analyzed before. We showed here that, while *cxcl12a* heterozygosity by itself does not lead to intraretinal pathfinding errors, the loss of one allele of *cxcl12a* in *shha* mutants significantly increases the intraretinal pathfinding phenotype compared to *shha* mutants. Intraretinal pathfinding errors are seen in nearly 100% of *cxcl12a* and *cxcr4b* mutants, while Hh pathway mutants only show pathfinding errors in roughly 50% of the embryos. We observed a low level of *cxcl12a* expression at the optic disc in *shha* mutants, and this residual expression may be sufficient to partly rescue the pathfinding phenotype in *shha* mutants. Using pharmacological Hh pathway inhibition starting at 1 hpf, we were able to induce pathfinding errors in up to 80% of the embryos. This may indicate that maternal *smo* mRNA deposition (Varga et al., 2001) is sufficient to partly rescue optic stalk patterning in *smo* mutants during early eye development even though *cxcl12a* expression was undetectable at 28 hpf in these embryos using *in situ* hybridization. Therefore, we propose that the increased pathfinding phenotype in *shha* embryos with only one allele of *cxcl12a* compared to *shha* mutants

may be explained by a further downregulation of Cxcl12a levels at the optic disc in these embryos. The low level of *cxcl12a* expression at the disc in Hh pathway mutants can either be explained by loss of specific gene expression or by a failure of cells differentiation in the optic stalk. Studies in mice showed that Hh signaling is necessary for astrocyte differentiation and maintenance in the optic stalk (Wallace and Raff, 1999; Dakubo et al., 2003, 2008). Our genetic interaction experiment, on the other hand, supports the model that axon pathfinding errors in *shha* mutants are due to the loss of guidance cue expression at the optic disc. This identifies the Hh signaling pathway as a regulator of *cxcl12a* expression in the zebrafish optic stalk. A similar indirect requirement for Hh signaling has been observed at the zebrafish chiasm, where Shh regulates the expression of *slit* guidance cues at the midline (Barresi et al., 2005).

We were unable to determine if expression of *cxcl12a* in the optic stalk in *shha* mutants is sufficient to rescue the pathfinding phenotype in these embryos due to technical limitations. In the future, it will therefore be interesting to further determine the molecular mechanism of Hh and chemokine pathway interaction; if Hh signaling similarly regulates other guidance cues involved in intraretinal axon guidance in zebrafish; and finally, whether this mechanism holds true in other organisms as well.

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CHAPTER 4

DISCUSSION

Summary

Over a century of research has been dedicated towards resolving the mechanisms involved in the formation of the complex vertebrate nervous system. A number of molecules have been shown to play important roles, some by patterning host tissue, some by regulating axon growth as guidance cues, and others, such as Shh, by acting in both roles. But still, many questions regarding the mechanisms of the development of the nervous system remain unresolved. One of these open questions is how Hh signaling regulates intraretinal axon pathfinding. While it is known that the loss of Shh leads to intraretinal axon misprojections, it is unclear whether this phenotype is due to mispatterning of ocular tissues or due to the loss of Shh as direct axon guidance cue. The aim of this dissertation was to address and resolve this question using zebrafish as a model system.

To characterize the intraretinal axon pathfinding errors in *syu* mutants, I developed a focal lipophilic dye injection method, which allowed me to visualize axon pathfinding of single bundles inside the eye. To directly address the question of how Shh regulates intraretinal axon pathfinding, I then determined the spatial and temporal requirement for Hh signaling in this process. My results revealed an indirect role for Shh in regulating tissue patterning but not in direct axon guidance. By investigating the mechanism downstream of Hh signaling, I found a novel interaction between the Hh and chemokine signaling pathways for the guidance of RGC axons out of the eye.

With this work, I provide important knowledge about the mechanisms of axon guidance by revealing a novel interaction between two signaling pathways in the process of axon pathfinding.

Characterizing the intraretinal axon pathfinding phenotype in *syu* mutants

A number of genetic tools are available to visualize retinal axons in zebrafish. Transgenic fish lines labeling RGC bodies and axons include the *isl2b:GFP* transgene, which was generated by a former graduate student in our group (Pittman et al., 2008). I used *isl2b:GFP* transgenic fish to analyze the global RGC projection phenotype seen in *syu* mutants. I found that in contrast to wt embryos, where all RGC axons exit the eye, axons misproject inside the eye in roughly 50% of the *syu* embryos. In addition, I found that the number of axons affected within a single eye is highly variable, ranging from only a few misguided axons up to all axons misprojecting inside the eye.

The *isl2b* transgene allows one to see all or the majority of the RGC projections as a group, but it does not allow the resolution of single axonal projections or even subregions of the retina.

To characterize the intraretinal pathfinding errors in more detail, and specifically to answer the questions of where within the retina the misguided axons originate and where along their pathway the errors occur, I was in need of a labeling method which allows one to visualize thin axon bundles inside the retina. I therefore revived a focal dye labeling method, which generates a minimal spill of dye inside the eye and labels single axonal bundles. This method was originally developed by Torsten Trowe and is described in his dissertation (Analyse von Mutationen mit Einfluss auf die topographische Ordnung von Axonen im retinotektalen System des Zebrabärblings, Danio rerio. PhD Thesis, Eberhard-Karls-Universität Tübingen, Tübingen, Germany, 2000). This technique uses

lipophilic dye coated needles, which are inserted quickly into the RGC layer of a fixed embryo embedded in agarose. The dye is allowed to diffuse along axonal shafts before the trajectories can be visualized. This method has been published as part of 'Methods in Cell Biology; The Zebrafish: Cellular and Developmental Biology, Part A' (Poulain et al., 2010) written by our group and is included as a reprint in Chapter 2 of this dissertation.

With a labeling method in hand, that fulfilled all the necessary requirements, I was able to characterize the intraretinal axon pathfinding phenotype in *syu* mutants. While focal dye injections in wt embryos show axon bundles projecting towards the optic disc, where they turn and exit the eye through the optic nerve, injections in *syu* embryos revealed that RGC axons from all quadrants of the eye can undergo pathfinding errors and that misguided axons preferentially project posteriorly within the retina, while anterior projections occur but are not seen as commonly.

In wt fish, RGC axons project in radial fascicles towards the optic disc where they all converge and leave the eye as one big bundle. The bundling of axons is thought to be mediated by cell adhesion molecules expressed along the axon shafts (Ott et al., 1998). In *syu* mutants, bundling still occurs and misprojecting axons often form thick bundles inside the eye. In case of anterior and posterior projecting axons in the same eye, the two axon bundles seem to project along each other, but in opposite directions. These findings indicate that while axon pathfinding out of the eye is defective in *syu* mutants, cell adhesion is not affected.

The analysis of *syu* axon projections revealed that axons project towards the optic disc in the same manner as it is seen in siblings, traveling along the basal lamina between

the RGC layer and the lens, where extracellular matrix molecules, such as *laminin*, are proposed to promote axon outgrowth (Smalheiser et al., 1994). While wt axons turn after reaching the optic disc to exit the eye through the optic nerve, *syu* axons fail to turn and instead continue growing inside the eye. These findings suggest that the axon pathfinding errors seen in *syu* mutants are due to a failure to turn into the optic stalk, while pathfinding towards the optic disc is normal.

In conclusion, this phenotypic analysis revealed that the errors in *syu* mutants can affect axons from all quadrants of the eye equally and occur locally at the optic disc. Therefore, we hypothesized that the axon pathfinding errors are due to the lack of one or more guidance cues localized at the optic disc, which are necessary to guide axons into the optic stalk and out of the eye.

Shh regulates intraretinal axon pathfinding indirectly

After analyzing the intraretinal pathfinding phenotype, I then wanted to clarify the mechanism of how Shh regulates retinal pathfinding. The experiments conducted to answer this question are described in Chapter 3 of this dissertation.

Previous research implicated Shh both in tissue patterning acting as a morphogen and in axon guidance acting as a direct guidance cue on axons themselves (see Chapter 1 'Introduction'). The pathfinding phenotype in *syu* mutants could therefore be the result of mispatterning of ocular structures or the lack of Shh as a guidance cue.

Analysis of the mRNA expression patterns of Hh pathway genes revealed that both ligand and receptor components are expressed at the anterior midline during early ocular patterning events, that ligand components are strongly expressed in the optic stalk

shortly before RGCs start to differentiate, and that all components analyzed show expression in the RGC layer during the time of axon pathfinding. In addition, I found that mRNAs are localized in the optic nerve, indicating that these may be transported along axons towards the growth cone. This could suggest that Hh pathway genes are locally translated in the growth cone during axon outgrowth.

In summary, the expression patterns and cellular localizations of Hh pathway mRNAs could suggest both a role in patterning of ocular structures and a role in direct axon guidance.

I performed retinal cell transplants to address the question of whether Hh pathway components act cell autonomously or non-cell-autonomously in intraretinal axon pathfinding. I found that both Shha and Smo act non-cell-autonomously in intraretinal axon pathfinding. This finding was a first indication of a role for Hh signaling in tissue patterning but not direct axon guidance.

Our lab previously studied the role of pioneer (early born) RGCs on the guidance of follower (later born) RGC axons. The results showed that inhibition of pioneer RGCs leads to pathfinding errors in follower axons, and that this phenotype can be rescued by resupplying central, pioneer, RGCs. In addition, this study showed that pathfinding errors of *robo2* mutant RGCs can partially be rescued by supplying wt pioneer RGCs (Pittman et al., 2008). These findings raised the question whether pioneer-follower interactions could possibly mask a cell-autonomous effect in my cell transplants. Therefore, I inhibited RGC formation by *ath5* MO injections. This approach allowed me to analyze donor RGC projections in an environment free of host, and therefore pioneer, axons. These transplants confirmed the non-cell-autonomous role of Smo in RGC axon

guidance, supporting my previous finding that Hh signaling does not act in direct axon guidance of RGC axons inside the zebrafish eye.

I used pharmacological inhibition of the Hh signaling pathway in a temporally controlled manner. Treatment starting at 1 hpf and 10 hpf, as well as treatment stopped at 24 hpf resulted in the same percentage of embryos with errors, while treatment started at 16 hpf or later resulted in fewer embryos with errors compared to continuous treatment starting at 1 hpf. This indicates that the Hh signaling pathway acts mostly between 10 and 16 hpf for correct axon pathfinding after 28 hpf. This early developmental time window signifies the time of optic vesicle patterning. The gradual decrease of embryos with pathfinding errors with treatments starting at 16, 24, and 28 hpf, suggests that the Hh signaling pathway is still involved in eye patterning after 16 hpf but with less impact on subsequent axon pathfinding. The temporal inhibition of Hh signaling therefore suggests that the pathway acts during early ocular tissue patterning but not during active axon outgrowth to regulate axon pathfinding inside the eye. This again supports an indirect role for Hh signaling in intraretinal axon pathfinding.

In conclusion, the spatial and temporal analysis of the requirement of Hh signaling strongly suggests that Shh regulates intraretinal axon pathfinding indirectly through patterning of the eye but not directly as an axon guidance molecule.

My findings stand in contrast to results published using chicken and mouse as model to study the role of Hh signaling in retinotectal pathfinding (Kolpak et al., 2005; Sánchez-Camacho and Bovolenta, 2008). The study using injection of cyclopamine into the vitreal space of the chicken retina proposed that late inhibition of Hh signaling is sufficient to induce intraretinal axon pathfinding errors (Kolpak et al., 2005). Using a

genetic approach, the mouse study proposed that Hh signaling acts cell autonomously in the proximal pathway (inside the eye), while it has a non-cell-autonomous effect in the distal pathway (at the chiasm). What are possible reasons for this inconsistency? First of all, these differences are likely due to species specific differences of the role of Hh signaling in intraretinal axon pathfinding. While the loss of Shh in all three organisms leads to intraretinal axon pathfinding errors, the role of the Hh signaling pathway may not be conserved. Studies in mice suggest that Shh released from RGCs is necessary for optic nerve astrocyte proliferation (Wallace and Raff, 2009; Dakubo et al., 2003, 2008) This role has not been described in zebrafish, but instead midline Shh seems to play the major role in patterning of the optic vesicle in this system (Ekker et al., 1995; Macdonald et al., 1995). In addition, both mouse and chicken retinas stop proliferating after the initial wave of retinal neuron differentiation. In zebrafish, on the other hand, new neurons are generated in the CMZ throughout life. This difference could suggest differing mechanisms in retinal development in these organisms and supports evolutionary differences for the role of Shh in regulating pathfinding of RGC axons inside the retina.

On the other hand, what are the technical differences that could have led to the differing results in the mouse, chicken, and my study? The mouse study used in-utero electroporations at E13.5, 2 days after the start of RGC differentiation, to inhibit the Hh pathway by delivering a Shh insensitive version of *ptch1*, which acts in a dominant negative manner. At this timepoint eye patterning should be complete. These injections resulted in transfected RGC axons with pathfinding defects inside the retina. Because nonelectroporated RGCs were not counterstained in these experiments, it is unclear whether the dominant negative construct only affected electroporated cells or whether

other RGCs were also misguided. The fact that Hh pathway inhibition after the start of RGC differentiation (at E13.5) still results in misguided axons in the eye stands in clear contrast to my findings using SANT75 treatment at 28 hpf. The effect of SANT75 treatment at this late time-point resulted in only 5% of the embryos with intraretinal pathfinding errors, while roughly 80% were affected with early treatment. The stark difference in the penetrance of the effect led me to conclude that Hh signaling is not required for intraretinal axon pathfinding at this late time. The mouse retinas seem to exhibit only a few misguided axons compared to the thick axon bundles misguided after SANT75 treatment in zebrafish. Because the electroporation was only done at one timepoint it cannot be excluded that earlier targeting would have had stronger effects. Because the mouse study does not show any quantification of the results, I cannot compare the penetrance of the phenotype between my findings and the mouse results.

Cyclopamine injections at a time when RGC axon outgrowth just begins induced intraretinal axon pathfinding errors in chicken. This timing corresponds to my SANT75 treatment starting at 28 hpf, the time when Hh pathway inhibition barely induced any pathfinding errors. Early optic vesicle injections in chicken, on the other hand, did not produce any pathfinding errors. The lack of phenotype with early injections was explained by the fast diffusion of the compound, which limits the effectiveness of the drug for treatments for longer periods of time. The group concluded that Hh signaling acts directly on axon guidance at the time of axon outgrowth but not through patterning of the eye (Kolpak et al., 2005). Whether continuous treatment starting at an early time-point may induce pathfinding errors in chicken is unclear due to the experimental conditions used, but in this system, late treatment seems to be sufficient to induce

intraretinal axon pathfinding errors. This stands in clear contrast to my findings in zebrafish and is most likely due to a differing role of Hh signaling in intraretinal axon pathfinding in chicken and zebrafish.

Previously, it was mentioned that Hh pathway inhibition using cyclopamine treatment in zebrafish starting at 12 hpf or 23 hpf induced intraretinal axon pathfinding errors (Kay et al., 2001), such as I observed using SANT75 application. No quantification of the results was given in this study and it is therefore not possible to evaluate whether the penetrance of the phenotype seen using the later treatment was different from early treatment or how the results compare to my SANT75 treatment. But nonetheless, the results from the zebrafish study supports my finding that early inhibition of the Hh signaling pathway in zebrafish induces intraretinal axon pathfinding errors.

While no definite conclusion can be drawn regarding the differences in the results found in the mouse and chicken studies compared to my findings, the indirect role for Hh signaling in intraretinal axon pathfinding in zebrafish is supported by my experiments determining both the spatial and temporal requirement for the Hh pathway. Therefore, the results found using other organisms may be due to the experimental approach chosen or may likely reflect an actual species specific difference in the mechanism of intraretinal axon pathfinding regulated by Shh. Future studies will therefore be needed to determine if these differences hold true.

The Hh pathway interacts with chemokine signaling for intraretinal pathfinding

If Hh signaling regulates retinal axon pathfinding through tissue patterning, we would expect that optic stalk markers are downregulated or missing in *syu* and *smo* mutants. To verify this hypothesis, I determined the expression of *pax2a*, a transcription factor expressed in neuroepithelial cells along the optic stalk, *netrin1a*, an axon guidance molecule expressed at the optic fissure, and *cxcl12a*, a chemokine ligand expressed in the optic stalk and disc.

Immunohistochemistry for Pax2a and *in situ* hybridization for *netrin1a* and *cxcl12a* revealed that all three markers are greatly downregulated in *syu* mutants and are lost completely in the optic stalk region in *smo* embryos. These findings confirm that the loss of Hh signaling leads to mispatterning of the optic stalk. The decrease of Pax2a expression in the stalk could be an indication for the lack of neuroepithelial cell formation, cell death, or could be due to the loss of *pax2a* expression specifically. Further analysis of cell morphology in the optic stalk in Hh mutants could give us an indication of whether there exists a failure of neuroepithelial cell differentiation.

Netrin-1 mouse mutants show a low degree of intraretinal axon guidance errors (Deiner et al., 1997). A previous graduate student in our group used *netrin1a* MO injections to determine whether *netrin1a* is involved in intraretinal axon guidance in zebrafish, but was not able to detect any misguided axons. I repeated this experiment and confirmed the lack of intraretinal axon guidance errors after downregulation of *netrin1a*. While zebrafish carry two *netrin1* homologs, *netrin1a* and *1b*, only *netrin1a* is expressed in the optic fissure, making it unlikely that *netrin1b* compensates for the loss of *netrin1a*

for the guidance of RGC axons out of the eye. The downregulation of *netrin1a* at the optic fissure in Hh pathway mutants, while an indication for optic stalk mispatterning, does not seem to account for the intraretinal axon pathfinding errors in zebrafish embryos. This finding supports a species specific difference in the molecular mechanism of intraretinal axon guidance between mouse and zebrafish. Loss of Hh signaling leads to downregulation of *netrin-1* expression at the optic disc in both species, but while *netrin-1* guides RGC axons out of the eye in mouse, this role maybe played by *cxcl12* in zebrafish.

In zebrafish, mutants for the chemokine receptor *cxcr4b* exhibit intraretinal axon guidance errors (Li et al., 2005). I also detected strong axon pathfinding errors in *cxcl12a* embryos. The pathfinding phenotypes are nearly indistinguishable from the errors found in Hh pathway mutants, but occur with an almost 100% penetrance in the chemokine pathway mutants compared to about 50% in Hh signaling mutants. Both *cxcl12a* and *cxcl12b* are expressed in the optic stalk, with *cxcl12a* localized in the more distal stalk and disc, while *cxcl12b* is localized in the proximal optic stalk. The intraretinal axon pathfinding phenotype in *cxcl12a* mutants, the attractive effect of Cxcl12b on RGC axons and the expression of both *cxcl12* paralogs in the optic stalk (Li et al., 2005) make this chemokine a prime candidate for a molecule regulating in intraretinal axon guidance in zebrafish.

For my study, I wanted to determine if the downregulation of *cxcl12a* in Hh pathway mutants is responsible for the intraretinal pathfinding errors in *syu* and *smo* embryos. First, I confirmed that not only Cxcl12b attracts RGC axons, but that Cxcl12a expression in the eye shows a similar effect. In addition, using *syu* host embryos, I found that Cxcl12a also attracts mutant RGC axons. One of our hypotheses of how Hh signaling

might regulate intraretinal axon pathfinding was that Shh may act as a competence factor for the attractive effect of *cxcl12a* at the optic disc. If this was the case, the loss of *shha* should eliminate the attractive effect of Cxcl12a. My results showed that Cxcl12a still attracts RGC axons and I therefore dismissed the possibility of Shh as a competence factor for Cxcl12a. On top of that, I did not only find the persistence of the attractive effect, but found that the number of *syu* hosts with RGC axons growing towards Cxcl12a expressing cells was greatly increased compared to wt hosts. Why could Cxl12a have a stronger effect in *syu* mutants? My *in situ* hybridization results showed a marked downregulation of *cxcl12a* mRNA in the optic stalk in *syu* mutants. Assuming that Cxcl12a at the optic disc attracts wt RGC axons into the stalk and out of the eye, we can imagine that this endogenously-expressed Cxcl12a counteracts the attractiveness of ectopic Cxcl12a expressed in the anterior eye. In *syu* mutants, where the endogenous *cxcl12a* level is decreased, the ectopic Cxcl12a may attract more axons towards the anterior eye. If this interpretation of my results holds true, it would support our model that the loss of *cxcl12a* expression at the optic disc is responsible for the intraretinal axon pathfinding errors in Hh pathway mutants.

To further analyze how the Hh and chemokine pathway regulate intraretinal axon pathfinding, I investigated a possible genetic interaction between these two pathways. I found a highly significant increase in the axon pathfinding phenotype in *syu*; *cxcl12a*/+ embryos compared to *syu* mutants that carry 2 wt *cxcl12* alleles. This result indicates a genetic interaction between the Hh and chemokine signaling pathways for the guidance of RGC axons inside the eye. What could be the mechanism of this interaction? Again, I use the mRNA expression results to support my hypothesis. If the loss of *syu* leads to a

downregulation of *cxcl12a*, then the additional loss of one allele of *cxcl12a* could further decrease the level of Cxcl12a at the optic disc. Now, while low levels of Cxcl12a may lead to intraretinal axon pathfinding errors in *syu* mutants, decreasing this level may induce the increase in the severity of the errors. This could also explain why the axon pathfinding phenotype in *cxcl12a* mutants, which probably lack gene expression at the disc completely, show a higher penetrance than *syu* mutants, which still show a low level of *cxcl12a* expression at the optic disc.

The non-cell-autonomous effect of Hh signaling and the requirement during eye formation for subsequent intraretinal axon pathfinding, the loss of optic stalk markers in Hh mutants, the attractive effect of Cxcl12a, and the genetic interaction between the two pathways, supports the hypothesis that Hh signaling regulates intraretinal axon pathfinding indirectly. In this model, Shh patterns ocular tissue by regulating the expression of downstream target genes such as *cxcl12a*. Cxcl12a may then act as direct axon guidance cue to ensure exit of RGC axons out of the eye or may modulate additional cues at the optic disc. Independent on the mechanisms downstream of Cxcl12a, I am proposing a novel mechanism in intraretinal axon pathfinding, where Hh signaling regulates chemokine signaling through pattering of the optic stalk.

What is the evidence for a role of Cxcl12a in intraretinal axon guidance? Loss of chemokine signaling induces intraretinal axon guidance errors in zebrafish and Cxcl12b was shown to have an attractive effect on RGC axons inside the eye (Li et al., 2005). This study supports a role in direct axon guidance. *In vitro* experiments, on the other hand, suggested that Cxcl12 modulates the repulsive effect of slit-2, semaphorin 3A and 3C but has itself no attractive effect on axons (Chalasani et al., 2003). This group proposed that

Cxcl12 acts through the receptor Cxcr4, to activate downstream signaling, which leads to elevation of cAMP. Using *in vivo* experiments in zebrafish, the same group later showed that Cxcl12 antagonizes Slit/Robo signaling in the retinotectal system (Chalasani et al., 2007). Here, axon pathfinding errors were rescued by MO-based knockdown of *cxcl12* in hypomorphic *robo2* mutants but not null mutants. This finding supported the hypothesis that Cxcl12 does not reduce the effect of Slits through attraction but modulation of the negative guidance response. In contrast, knockdown of *cxcl12* induced intraretinal axon guidance errors equally in both hypomorphic and null *robo2* mutants. This result cannot be easily explained by a modulatory effect of Cxcl12. Therefore, this group proposed that Cxcl12 acts by antagonizing other negative guidance molecules outside of the eye, but suggested that it may act as direct guidance cue inside the retina. The direct role in intraretinal axon guidance is in agreement with the results found by John Kuwada's group (Li et al., 2005) and with my own results presented here.

An interaction between the Hh and chemokine pathways has previously been proposed using an *in vitro* essay to analyze the proliferation rate of cerebellar granule cells (Klein et al., 2001). Here, the simultaneous application of Shh and Cxcl12a induced greater proliferation than Shh alone, while Cxcl12a by itself had no mitogenic effect. While this study revealed an interaction between the Hh and chemokine pathways, the mode of interaction seems clearly different from the mechanism I found for the guidance of retinal axon in the eye in zebrafish. My Cxcl12a misexpression results show that the chemokine pathway has an attractive role even without the presence of Shh, suggesting that here, Cxcl12a does not merely modulate the response of Shh but acts itself as direct

guidance cue or may potentially modulate the effect of other guidance cues at the optic disc.

Therefore, my work proposes a novel mechanism for the guidance of retinal axons out of the eye, revealing the regulation of the chemokine pathway, specifically *cxcl12a*, by Shh.

Conclusions and outlook

At first glance, the exit of RGC axons from the eye appears like a simple task compared to pathfinding across the midline or towards higher brain regions. But when giving these first steps inside the eye a closer look, we must recognize how just this short distance involves an array of separate pathfinding decisions that have to be taken correctly to allow further progression towards target tissues. The array of tasks involves formation of a single axon, extension of this axon towards the central retina, fasciculation with other axons, extension in the correct cellular layer, reaching the optic disc, turning at the disc, and eventually leaving the eye through the optic nerve. As described before, a number of molecules have been implicated in these first steps of pathfinding out of he eye. In summary, CSPGs, HS, and Slit-Robo signaling were implicated in regulating the directionality of axon outgrowth; cell adhesion molecules, such as Neurolin and L1, are important for axon fasciculation; extracellular matrix molecules like Laminin promote axon outgrowth in the optic fiber layer; EphB receptors seem to specifically prevent overshooting of the optic disc by dorsal axons; while Netrin-1 is implicated in short range signaling at the optic fissure to ensure exit from the eye, similar to the role of chemokine signaling at the optic disc (Ott et al., 1998; Ogata-Iwao et al., 2011; Deiner et al., 1997;

Snow et al., 1991; Birgbauer et al., 2000, 2001; Thompson et al., 2006, 2009; Li et al., 2005; Dakubo et al., 2003; Sánchez-Camacho and Bovolenta, 2008; Schauerte et al., 1998). Even though it was known that Shh mutants show intraretinal axon pathfinding errors when I started my dissertation project, it was unclear at the time, which aspect of intraretinal axon pathfinding was affected or what the mechanisms is through which Hh signaling controls retinal pathfinding.

My experiments suggest that the intraretinal axon pathfinding errors seen in Hh pathway mutants are due to the downregulation of *cxcl12a* at the optic disc. The rescue of the intraretinal axon pathfinding phenotype in *syu* mutants by re-expression of *cxcl12a* in the optic stalk or disc in *syu* mutants would strengthen this hypothesis. Possibly, we could find a partial rescue of the phenotype, indicating that *cxcl12a* is not the only axon guidance molecule, acting in guidance of RGC axons out of the eye, which is regulated by the Hh signaling pathway.

I concentrated on the role of Cxcl12a for my studies, but *cxcl12b*, a homologous gene, is also expressed in the optic stalk, proximal to *cxcl12a*, and was shown to have an attractive effect on RGC axons (Li et al., 2005). It is unclear at this time if these two genes function redundantly or if each homologue has its specific role for the guidance of RGC axons. Possibly, while Cxcl12a induces turning into the stalk, Cxcl12b could promote pathfinding further into the stalk to prevent axons from making a U-turn and growing back towards the retina. While Cxcr4b is expressed in RGCs and acts as receptor of Cxcl12a for intraretinal axon guidance, Cxcr7b, expressed in the optic stalk, is also known to act as receptor for Cxcl12a (Burns et al., 2006). In the zebrafish lateral line system, both Cxcr4b and 7b are necessary for migration of the primordium and Cxcr7b

was proposed to act as a sink to restrict the activity of Cxcl12a to tip cells (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). My preliminary analysis of a *cxcr7b* mutant, kindly provided by a member of the Piotrowski group, did not reveal any intraretinal axon guidance errors. This may indicate that *cxcr7b* is not involved in guidance of RGC axons out of the eye or could be the result of gene redundancy with *cxcr7a*. The expression pattern of *cxcr7a* has not been reported in detail but a previous study analyzing the role of chemokine signaling in olfactory placode formation may suggest expression in the optic stalk region at 24 hpf in zebrafish (Miyasaka et al., 2007). Combined inactivation of *cxcr7a* and *7b* may therefore reveal the role of these receptors for intraretinal axon guidance.

While I could not detect a role for *netrin1a* in guiding RGC axons out of the eye, its prime expression at the optic fissure during eye development and RGC outgrowth and the intraretinal axon pathfinding errors found in *netrin-1* mice make me wonder whether future experiments may uncover such a role in zebrafish. The use of genetic interaction studies, such as I conducted with the Hh and chemokine pathway, may allow us to reveal molecular mechanisms of intraretinal axon pathfinding which were previously not reported.

While the molecules mentioned above are all implicated in intraretinal axon pathfinding, it is unclear how these pathways interact and what upstream regulators coordinate their actions. Are other morphogens acting in patterning of the eye and regulating the expression of axon guidance molecules, next to Shh? How many genes involved in intraretinal axon pathfinding are regulated by Shh? Does Shh directly

regulate *cxcl12a* expression or what other genes belong in this signaling cascade? Is this mechanism unique to retinal axons or is it found in other axon pathfinding systems?

One complication in revealing genes involved in retinal axon guidance comes from the finding that follower axons are guided by pioneer RGCs (Pittman et al., 2008). Genes regulating axon guidance may be masked by these pioneer-follower interactions. A possibility to uncover such molecules is to inhibit cell adhesion by knocking-down cell adhesion molecules. This technique could reveal mechanisms, which specifically regulate follower axon guidance.

The approach used in the study mentioned above (Pittman et al., 2008) did not differentiate between the role of pioneer RGC bodies and axons. Possibly, pioneer cell bodies located close to the optic disc express attractive cues that guide follower axons towards the central retina through long range signaling. On the other hand, pioneer RGC axons could guide followers in a more contact based manner on the path towards the disc. Future experiments could aim to inhibit pioneer RGC axon formation while differentiation would remain unaffected. A possible candidate for a gene involved in axon formation is *stk11* (Barnes et al., 2007). This study showed that knockout of *stk11* led to failure of axon formation in mouse cortical neurons. In zebrafish, *stk11* is widely expressed during development, including the eye (Marshall et al., 2011). The knockdown of *stk11* using MO injections may therefore result in failure to differentiate RGC axons. This approach would allow us to clarify the role of pioneer RGC bodies versus axons in follower guidance, and the combination with genetic manipulations could reveal the molecules involved in this process.

I envision a future textbook clearly describing the mechanism of intraretinal axon guidance, outlining cues expressed by the optic disc and stalk or by pioneering RGCs; genes involved in directing axons towards the disc or keeping them in the correct layer; molecules with attractive or repulsive roles; as well as additional genes involved in inducing turning at the optic disc. And just as important as revealing the axon guidance cues involved, future research will reveal additional patterning genes, which act upstream of direct guidance molecules, and are therefore essential for the correct expression of the later, such as I have shown for Shh and Cxcl12a.

In conclusion, my studies revealed a novel interaction between the Hh and chemokine pathways for the guidance of RGC axons out of the eye in zebrafish. Both the applied methods and genetic mechanisms I revealed signify important steps in this field of research and open doors for future studies directed to resolve the mechanisms of axon guidance not only inside the eye but also for other axon tracts.

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